Inflammatory Wnt5A Signaling in Macrophages as Novel Target for Anti-inflammatory Action of Activated Protein C

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1. Preface

The work of this thesis was performed at the Department of Internal Medicine at the University Hospital of Zurich. The work is part of the project No. 3200B0-103945 of the Swiss National Science Foundation.

The aim of this work was to study the transcriptional response of monocytes during inflammation and define new targets for the anti-inflammatory action of activated protein C (APC).

The data is presented in form of the following publications or manuscripts submitted for publication:

1. **Analysis of the Inflammatory Transcriptome of Human Macrophages Revealed Unique Genes as Targets for the Anti-inflammatory Action of Activated Protein C**
   Pereira C, Bachli EB, Schaer DJ, Schoedon G
   Submitted manuscript

2. **Wnt5A/CaMKII signaling contributes to the inflammatory response of macrophages and is a target for the anti-inflammatory action of activated protein C and interleukin-10**
   Pereira C, Schaer DJ, Bachli EB, Kurrer MO, Schoedon G
   Arterioscler Thromb Vasc Biol, 2008. 28(3): p. 504-510.

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2. Abbreviations

APC	activated protein C
COX-2	cyclooxygenase-2
CSF	colony stimulating factor
DIC	disseminated intravascular coagulation
EC	endothelial cells
EGF	epidermal growth factor
EPCR	endothelial protein C receptor
ICU	intensive care unit
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
PAI-1	plasminogen activator 1
TFPI	tissue factor pathway inhibitor
PAR-1	protease activated receptor-1
PAR-2	protease activated receptor-2
PC	protein C
PCI	protein C inhibitor
PCP	planar cell polarity
PROWESS	Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis study group
rAPC	recombinant activated protein C
TAFI	thrombin-activatable fibrinolysis inhibitor
TM	trombomodulin
tPa	tissue plasminogen activator

3. Zusammenfassung

Bei schwerer systemischer Entzündung und Sepsis finden grundlegende Veränderungen an Komponenten des Gefässsystems statt, insbesondere die entzündliche Aktivierung von Zellen der Gefässwand wie Endothel und glatte Muskelzellen sowie die Aktivierung von Blutzellen wie Monozyten undThrombozyten. Es kommt zur Ausschüttung von entzündungsfördernden Mediatoren wie Stickstoffmonoxid NO, Zytokinen und Chemokinen wie TNFα, IL-1, IL-6, sowie Tissue Factor (TF). Diese Faktoren sind verantwortlich für Blutdruckabfall, Fieber und disseminierte intravasale Gerinnung, was schliesslich zu Schock und Multiorganversagen mit hoher Mortalität führt. Ziel unserer Forschung ist es, die Entzündungsprozesse in den beteiligten vaskulären Zellsystemen zu verstehen und damit Möglichkeiten einer anti-inflammatorischen Intervention aufzuzeigen.

Das Hauptziel der vorliegenden Dissertation war es, die Antwort von Blutmonozyten auf inflammatorische Stimulation mit IFNγ in Kombination mit einem mikrobiellen Trigger wie Lipopolysaccharid (LPS) auf transkriptioneller Ebene zu untersuchen, um neue Zielgene für anti-inflammatorische Interventionen mit aktiviertem Protein C (APC) zu finden. APC ist ein körpereigener Gerinnungshemmer mit möglicherweise anti-inflammatorischen Eigenschaften. Durch Analyse des Genexpressionsprofils von entzündlich aktivierten Monozyten konnten wir das gesamte Spektrum von Genen, welche an der Entzündungsantwort beteiligt sind, definieren. Eine funktionelle Clusteranalyse zeigte eine signifikante Ueberrepresentation von entzündungsassoziierten Genen (induzierte Gene) und Genen welche mit Lipidstoffwechselprozessen assoziiert sind (supprimierte Gene). Die anti-inflammatorische Wirkung von APC konnte erstmals in entzündlich aktivierten Monozyten bestätigt werden. Ausserdem konnten wir Signalmoleküle und Transkriptionsfaktoren, welche bisher in Monozyten nicht bekannt waren oder nicht mit Entzündungsprozessen in Verbindung gebracht werden konnten, als Angriffsziele von APC definieren. Unter anderen fanden wir, dass APC den Acetylierungszustand von Histonen moduliert und somit eine Rolle im Chromatinremodeling spielt. Damit wird die Transkription bestimmter Genabschnitte reguliert, die bei einer Entzündungsantwort von Monozyten wichtig sind.

Diese Resultate vermitteln ganz neue Einblicke für therapeutische Möglichkeiten bei schwerer systemischer Entzündung und Sepsis.

Eines der durch APC am stärksten regulierten Gene ist Wnt5A. In Makrophagen wurde Wnt5A erst kürzlich als ein wichtiger Faktor des durch Toll-Rezeptoren vermittelten inflammatorischen Signalwegs erkannt. Wir sahen, dass Wnt5A durch ein Tollrezeptor vermitteltes Entzündungssignal stark heraufreguliert wird. Wnt5A wiederum induzierte die Gene für die inflammatorischen Zytokine IL-6, IL-8, und MCP-1 über einen Ca2+/Calmodulin abhängigen Proteinkinase II (CaMKII) Signalweg. Dieser Signalweg konnte durch solubles Frizzled-related Peptide 1 (sFRP1), ein lösliches Peptid welches den Wnt5A-Signalweg moduliert, komplett gehemmt werden. Die Tatsache, dass sFRP1 auch die LPS/IFNγ induzierte Phosphorylierung von CaMKII hemmt, deutet auf einen Zusammenhang zwischen entzündungsvermittelter Freisetzung von Wnt5A und LPS/IFNγr induzierter Aktivierung der Makrophagen hin. Wir konnten zeigen, dass die Expression von Wnt5A ein hochkonservierter Vorgang bei der Makrophagenaktivierung ist und ein breites Spektrum von Tollrezeptor-Liganden bei der Auslösung systemischer Entzündungsreaktion beteiligt sind. Wir konnten weiterhin zeigen, dass die anti-inflammatorisch wirksamen Mediatoren IL-10 und APC diesen Effekt stark beeinflussen. In Seren von Patienten mit schwerer systemischer Entzündung oder septischem Schock fanden wir grosse Mengen immunpräzipitierbares Wnt5A, ein weiterer Hinweis auf eine aktive Rolle von freigesetztem Wnt5A in der Pathophysiologie von systemischen Entzündungsreaktionen bei Sepsis. Dass Wnt5A ein sezerniertes Entzündungsprotein ist und sein Signal durch klassische Rezeptor-Ligand-Interaktion weitergibt, ermöglicht eine Beeinflussung seiner Aktivität *in vivo*. Eine Möglichkeit hierfür wäre die therapeutische Anwendung löslicher Wnt5A neutralisierender Moleküle, wie wir sie in unserer Arbeit verwendet haben, um den entzündlichen Wnt5A-Signalweg zu hemmen.

4. Summary

During systemic inflammation and sepsis, different components of the vascular system are altered, especially cells of the vessel wall such as endothelium and smooth muscel cells are inflammatory activated, as well as blood cells such as monocytes and platelets. Thereby pro-inflammatory mediators such as nitric oxide NO, cytokines, and chemokines such as TNFα, IL-1, IL-6, and tissue factor were released. These factors are responsible for reduced blood pressure, fever, and disseminated intravasal coagulaton, leading to shock, multiorgan failure and death. The aim of our research is to define the processes involved in the inflammatory response of the vascular cell systems and to find targets for anti-inflammatory intervention.

The main goal of this thesis was to study the transcriptional response of monocytes after treatment with inflammatory stimuli (stimulation with interferon (INF) followed by exposure to a microbial trigger like lipopolysaccharide (LPS)) and define new targets for the anti-inflammatory action of activated protein C (APC), an endogenous anti-coagulant with possible anti-inflammatory properties. By gene expression profiling analysis of inflamed macrophages, candidate genes involved in inflammatory responses were defined. Functional clustering of genes significantly regulated by inflammatory stimuli showed a significant overrepresentation of genes involved in the inflammatory response (upregulated genes), and genes associated with lipid metabolic processes (downregulated genes). We were able to confirm the anti-inflammatory action of APC in inflamed macrophages, and define signaling molecules and transcription factors not assigned to inflammatory responses as targets for APC. Among others, we found genes that modulate the acetylation and deacetylation of histone that were regulated by APC, indicating that APC could be involved in chromatine remodeling and thus in transcription regulation of genes that play a role in the inflammatory response of macrophages. These results provide novel insights for therapeutic strategies using APC in systemic inflammation and sepsis.

Among the genes most prominently regulated by APC was Wnt5A, a gene that recently emerged to be an important factor for the Toll-mediated inflammatory signaling in macrophages. We have found that Wnt5A was consistently upregulated via Toll-like receptor activation in inflamed monocytes. Wnt5A in turn upregulated expression of the pro-inflammatory genes IL-6, IL-1β, IL-8, and MCP-1 via Ca2+-

calmodulin–dependent protein kinase II (CaMKII) signaling. This effect was completely abrogated by sFRP1, a soluble modulator of Wnt signaling. Our finding, that sFRP1 also inhibited the LPS/IFN-γ induced CamKII phosphorylation, implied a causal link between Wnt5A secretion triggered by inflammation and LPS/IFN-γ induced macrophage activation. We showed that expression of Wnt5A constitutes a highly conserved response to macrophage activation triggered by a wide array of Toll ligands involved in initiation of systemic inflammation**.** Attenuation of this effect was achieved by the anti-inflammatory mediators IL-10 and APC. High levels of immunoprecipitatable Wnt5A in sera from patients with severe sepsis provided direct evidence of an active role for secreted Wnt5A in the pathophysiology of the systemic inflammatory response during sepsis. The fact that Wnt5A is a secreted inflammatory protein and that Wnt5A signaling involves classical receptor–ligand interactions raises the possibility of modulation of Wnt5A activity *in vivo*. Therapeutic application of soluble Wnt5A neutralizing molecules, as applied in our work, thereby represents just one possibility for achieving *in vivo* suppression of inflammatory Wnt signaling.

5. Introduction

5.1 The vascular system

The vascular system is constituted by a complex and articulate network, e.g. arteries, arterioles, venules and veins, that requires a high degree of coordination between different elemental cell types. The endothelial cells (EC) play a pivotal role in the vascular homeostasis being located at the interface between blood and the underlying smooth muscle cells. EC regulate complex functions compliant to specific necessities in time and location [1].

5.1.1 Immunovascular communication

Inflammation is generally defined as the localized response to cellular or tissue injury [2]. It is clear that each type of inflammation is not a simple linear cascade but rather a complex highly orchestrated and fine-tuned process involving interactions between many different types of cells, soluble mediators and tissue matrix. Some mediators initiate or amplify an inflammatory process some modulate it or cause it to subside and a number probably can do either depending on the context in which they are acting. Many different cell types may participate in inflammatory processes including among others, phagocytic cells, dendritic cells, mast cells and basophils, endothelial cells, smooth muscle cells, platelets and lymphocytes. Even larger numbers of intercellular signaling molecules participate in various inflammatory processes, including immunoglobulins, arachidonic acid metabolites complement components, the intrinsic clotting system and coagulation factors, fibrinolytic molecules and a large number of cytokines and chemokines.

Mononuclear cells play a key role in immunovascular communication by releasing the classical pro-inflammatory cytokines interleukine (IL)1β, IL6 and tumor necrosis factor (TNF)α, but in addition an array of other cytokines, chemokines, lipid mediators and reactive oxygen species. Most of these products have multiple targets and cause effects through several parallel mechanisms. Furthermore, many macrophage products are involved in the regulation of each other. Thus TNFα upregulates tissue factor (TF), IL18 induces interferon (INF)γ, which in turn further activates macrophages, while IL10 is a global suppressor of macrophage function. Bacterial lipopolysaccharide (LPS) in complex with LPS-binding protein (LBP) binds to

monocytes via CD14 and toll-like receptor 4 (TLR4). This results in activation of pro-inflammatory and coagulation pathways. Pro-inflammatory cytokines (e.g. TNFα, IL1β) act in autocrine and paracrine loops to further activate monocytes and endothelial cells by upregulating adhesion molecules, cytokines, chemokines and growth factors. TNFα and IL1β can also induce apoptosis of monocytes and endothelial cells. If left uncontrolled, these highly complex and tightly regulated networks can lead to widespread inflammation, thrombosis, cell death, organ failure, and ultimately death [3].

5.1.2. Inflammation and sepsis

In contrast to the normal inflammatory response, sepsis is a devastating condition characterized by systemic activation of inflammatory and coagulation pathways in response to microbial infection of normally sterile parts of the body [4]. Microbial invasion originates from a breach of integrity of the host barrier, either physical or immunological. Sepsis is the leading cause of death in non-coronary intensive care unit (ICU) patients and is a leading cause of morbidity and mortality in the Western world [5]. There are several important themes in our current understanding of sepsis pathophysiology. First, it is rare for the initial infection to be the cause of mortality; rather, mortality is the result of the body's response to infection. Although activation of the innate immune system is generally protective, an excessive or insufficiently controlled immune response may harm the host through a maladaptive release of inflammatory mediators. Second, monocytes and endothelial cells play a key role in modulating the host response to infection. As a first line of defence, monocytes recognize microbial products such as LPS through pattern recognition receptors (e.g. Tolllike receptors). The interaction of pathogens with monocyte receptors activates both the inflammatory and coagulation pathways. On the inflammation side, activated monocytes release inflammatory mediators that function in autocrine or paracrine loops to further activate monocytes and/or endothelial cells. The endothelium responds to these mediators with structural changes, such as cytoplasmic swelling and detachment and importantly, also with functional changes, such as the expression of adhesion molecules, resulting in increased platelet adhesion and leukocyte trafficking [1, 6]. On the coagulation side, activated monocytes and endothelial cells express TF on their cell surface, the "spark" that triggers blood

clotting [7]. Thus, monocytes and endothelial cells have the potential to inflict “collateral damage” to host tissues. A third theme in our understanding of sepsis pathophysiology is that inflammation, coagulation, and apoptosis are intimately linked. If left uncontrolled, the vicious cycles of inflammation and coagulation can lead to widespread inflammation, thrombosis, cell death, organ failure, and ultimately death [8, 9].

5.1.3. Inflammation, coagulation and sepsis

Under normal circumstances, the natural anticoagulant mechanisms provide a potent defence against thrombotic complications. Inflammatory mechanisms shift the haemostatic balance to favour the activation of coagulation and, in the extremes, either disseminated intravascular coagulation or thrombosis. Inflammatory mediators can elevate platelet count, platelet reactivity, downregulate natural anticoagulant mechanisms, initiate the coagulation system, facilitate propagation of the coagulant response and impair fibrinolysis. Similarly, clotting can increase the inflammatory response both by releasing mediators from platelets and by activating cells, thereby promoting cell–cell interactions that increase the inflammatory responses [10, 11].
It is now well established that in sepsis, systemic inflammation invariably leads to activation of the coagulation system and inhibition of anticoagulant mechanisms and fibrinolysis. Activation of coagulation and subsequent fibrin deposition are essential parts of the host defense against infectious agents in an attempt to contain the invading microorganisms and the subsequent inflammatory response [12]. An exaggerated response, however, can lead to a situation in which coagulation itself contributes to disease in its most severe form causing microvascular thrombosis and organ dysfunction, a syndrome known as disseminated intravascular coagulation (DIC) [13]. It is becoming increasingly clear, that vice versa, components of the coagulation system are able to markedly modulate the inflammatory response [10, 11].

5.2. The protein C pathway

There are three major anticoagulant mechanisms that control the blood clotting process: tissue factor pathway inhibitor (TFPI), the heparin-antithrombin pathway and

the protein C (PC) anticoagulant pathway. During inflammation-induced activation of coagulation, the function of all 3 pathways can be impaired. There is a differential pattern of expression of the various anticoagulant pathways in different vascular beds; for example, in the coronary circulation, TFPI is mostly expressed in microvessels, whereas the protein C system is more universally present.

The protein C system exerts its anticoagulant effect by regulating the activity of FVIIIa and FVa, the cofactors in the tenase and prothrombinase complexes, respectively. Protein C is a vitamin K-dependent multi-domain protein composed of a light and a heavy chain, the two chains being disulfide-linked. A vitamin K-dependent domain containing c-carboxy-glutamic acid (Gla) residues and two epidermal growth factor (EGF)-like domains occupy the light chain. The heavy chain contains a short activation peptide and a serine protease domain [14]. Vitamin K-dependent post-translational carboxylation of glutamic acid residues generates the Gla residues. These residues are important for calcium binding to the Gla-domain and for the proper folding of the domain. The correctly folded Gla-domain binds negatively charged phospholipid membranes [15]. Protein C circulates as a proenzyme to an anticoagulant serine protease and is activated by thrombin bound to the endothelial membrane protein thrombomodulin (TM). Activated protein C (APC) cleaves and inhibits coagulation cofactors FVIIIa and FVa, which result in downregulation of the activity of the coagulation system [8]. The protein C pathway comprises multiple proteins involved in the different reactions. Thus, there are proteins affecting the activation of protein C by the Thrombin–TM complex, cofactor proteins that modulate the proteolytic activity of APC and serine protease inhibitors that inactivate APC. The endothelial protein C receptor (EPCR) stimulates the Thrombin–TM-mediated activation of protein C on the endothelial cell surface [16]. The two cofactors, protein S and the intact form of FV, enhance the anticoagulant activity of APC. Protein S is sufficient for inactivation of FVa, whereas regulation of FVIIIa in the tenase complex requires the synergistic contribution of protein S and FV. Protein S is a vitamin K-dependent plasma protein, which in human plasma to 60–70% is bound to the complement regulator C4b-binding protein (C4BP). The free form of protein S function as APC cofactor, whereas the complexed form does not [17]. Protease inhibitors such as the protein C inhibitor (PCI), a1-antitrypsin, and a2-macroglobulin inhibit APC in plasma. The inhibition is relatively slow and the half-life of APC in circulation is approximately 20 min [18]. Recently, protein C has been shown not only

to express anticoagulant activities but also to function as an anti-inflammatory and anti-apoptotic factor. Moreover, APC has been found to be useful as a therapeutic agent in the treatment of sepsis with the unique combination of anticoagulant, anti-inflammatory and anti-apoptotic properties of APC presumably being important [19].

5.2.1. Anticoagulant and profibrinolytic functions of APC

APC, a plasma serine protease, is best known for its ability to inhibit blood clot formation [8, 20]. APC acts as an anticoagulant by degrading clotting factors Va and VIIIa, thereby attenuating the coagulation cascade. *In vivo*, APC is generated in the circulation “on demand” from its inactive precursor PC. The protease that triggers the conversion of PC to APC is thrombin. Briefly, vascular injury or inflammatory cytokines/endotoxin initiate the coagulation cascade, ultimately resulting in thrombin generation and blood clot formation. Excess thrombin then complexes with TM, a receptor on endothelial cells. The thrombin-TM complex rapidly converts PC to its active form APC. An accessory factor, EPCR, binds circulating PC and presents it to the thrombin-TM complex, which augments APC generation by 10– to 20-fold. Recently, the anticoagulant activities of APC have been shown to extend beyond its ability to degrade factors Va and VIIIa. It has been shown that APC inhibits TF expression and activity on U937 cells and on blood monocytes [21, 22].

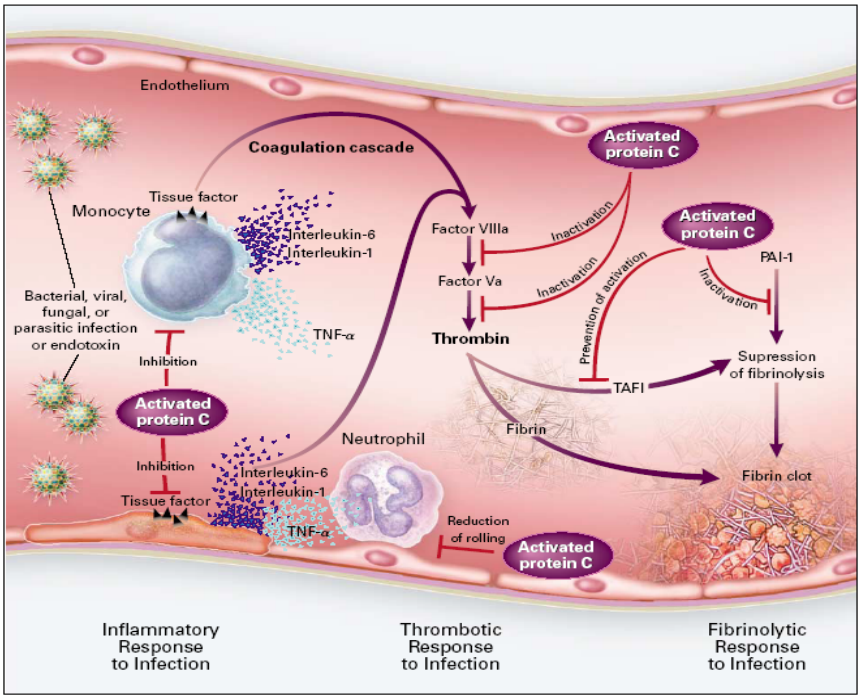

Figure 1. Proposed actions of Activated protein C in modulating the systemic inflammatory, procoagulant, and fibrinolytic host responses to infection. Adapted from [23].

In human blood monocytes challenged with LPS, recombinant human activated protein C (rAPC) inhibits TF antigen expression levels and TF procoagulant activity [21]. In human U937 monoblastic promyeloid leukemia cells, APC inhibits TF expression in phorbol ester stimulated cells in an EPCR-dependent manner [22]. These studies suggest that part of the protective effect of rAPC therapy may reflect the ability of rAPC to dampen the procoagulant potential of activated monocytes. APC also plays an important role as a profibrinolytic agent. Patients with severe sepsis have significantly increased levels of plasminogen activator inhibitor (PAI)-1, which has demonstrated to be predictive of poor prognosis [24]. APC neutralizes PAI-1 activity thereby preventing the inhibition of tissue plasminogen activator (tPa) by PAI-1 and promoting clot lysis. Furthermore, the inhibition of thrombin generation by APC limits the activation of thrombin-activatable fibrinolysis inhibitor (TAFI) and diminishes the inhibition of fibrinolytic pathways [25].

5.2.2. Anti-inflammatory action of APC

APC exerts direct anti-inflammatory effects on several cell types important in sepsis pathophysiology. In endothelial cells, APC modulates the p50/p52 subunits of the nuclear factor κB (NFκB) transcription factor complex and reduces the binding of the p65 subunit to DNA [26]. APC suppresses expression of endothelial cell adhesion molecules such as vascular adhesion molecule (VCAM)-1, inter-cellular adhesion molecule (ICAM)1, and E-selectin in TNF-α stimulated cells [26]. Downregulation of endothelial cell adhesion molecules by APC reduces E-selectin-dependent rolling of leukocytes, thereby limiting diapedesis. APC also upregulates IL-6 and IL-8 in endothelial cells, which is hypothesized to attenuate the inflammatory response via inhibition of neutrophil migration and accumulation. Induction of monocyte chemoattractant protein (MCP)-1 in endothelial cells by APC may facilitate endothelial cell migration and proliferation, thereby accelerating wound healing [27]. Furthermore, APC upregulates endothelial cyclooxygenase (COX)-2 protein and mRNA expression in an EPCR and protease activated receptor (PAR)- 1-dependent manner. The upregulation of COX-2 levels by APC and release of prostacyclin (PGI2) may provide further benefit in sepsis by improving blood flow [28]. In blood monocytes and in the monocytic cell line THP-1, APC inhibits LPS-induced activation of NFκB resulting in the downregulation of pro-inflammatory cytokines [29, 30]. In

addition, rAPC inhibits the release of macrophage inflammatory protein-1-alpha (MIP-1-α) from THP-1 cells [31] and inhibits NFκB activation and MIP-1-α production from monocytes from septic patients [32]. Furthermore, rAPC upregulates the anti-inflammatory cytokine IL-10 in blood monocytes, which might shift the balance of cytokines to promote anti-inflammatory effects [21]. APC may also exert anti-inflammatory effects through inhibition of leukocyte chemotaxis [33-35]. In neutrophils, both PC and APC inhibit chemotaxis induced by IL-8, antithrombin, formyl-Met-Leu-Phe, or C5a [33]. In lymphocytes, PC and APC inhibit cell migration, an effect independent of direct PAR-1 or PAR-2 involvement [34]. Interestingly, in the studies mentioned, PC and APC were equally effective in inhibiting chemotaxis, and the effects were EPCR-dependent. In-vivo data further supports the anti-inflammatory properties of APC. In baboons infused with lethal doses of *E. coli*, exogenously added APC reduces coagulopathy and organ dysfunction, while inhibition of generated APC results in elevated levels of inflammatory cytokines [36]. Likewise, the PROWESS trial revealed that rAPC infusion reduced levels of IL-6 [23]. Studies in an endotoxemia rat model found that rAPC treatment attenuated the adherence of leukocytes to the endothelium in the intestinal wall and improved microvascular perfusion [37]. In a human model of endotoxin-induced pulmonary inflammation, rAPC treatment reduced neutrophil accumulation in the pulmonary airspace and prevented neutrophil chemotaxis as compared to placebo following endotoxin administration [38]. A recent study showed that APC and IL10 act as anti-inflammatory agents by interfering with Wnt5A signaling and the general inflammatory response of human macrophages to LPS and INFγ [39].

5.3. The Wnt pathway

The Wnts comprise a large family of highly conserved growth factors that are responsible for important developmental and homeostatic processes throughout the animal kingdom [40]. Their implication in a wide array of developmental events and human diseases has made Wnts and their signaling pathways the subject of intense investigation over the last two decades [41-43]. Membership in the Wnt family is defined by amino acid code sequence rather than functional properties. The mammalian genome encodes 19 Wnt proteins and 10 Frizzled (Fz) seven-pass transmembrane receptors, which suggests that, in theory, 190 potential Wnt-Frizzled combinations exist. It has been proposed that Wnts activate a number of different signaling pathways [44], each of which has been shown to intersect with numerous other intracellular signal transduction pathways. That activation of some, but not all, Wnt pathways requires co-receptors such as low-density lipoprotein receptor–related protein 5 (LRP5) and LRP6 further complicates matters. It now appears that LRP5/6 has a second function: it binds to a molecule that counteracts Wnt [45]. That molecule is called Dickkopf (Dkk), meaning 'fat head', because it has the remarkable activity of promoting head formation in vertebrates [46]. Dkk and Wnt do not have similar amino-acid sequences, suggesting that Dkk does not act as a competitive inhibitor by binding to Frizzled or LRP5/6 in Wnt's place. In fact, it does not bind to Wnt or to Frizzled at all. Mao *et al.[45]* found that DKK binds to LRP5/ 6 through a part of that is not needed for interactions with either Wnt or Frizzled. While Dkk1 acts as a pure inhibitor of Wnt/β-catenin signaling, Dkk2 can either activate or inhibit the canonical pathway, depending on cellular context [47]. At low and high LRP5/6 levels Dkk2 functions as a Wnt inhibitor or activator, respectively. The ability of Dkk2 to act either as agonist or antagonist of Wnt/LRP6 signaling can also be regulated by the Dkk receptor Kremen 2 (Krm2), which invariability turns Dkk2 into a Wnt inhibitor [48]. Although the majority of work in the field to date has focused on β-catenin-dependent, or canonical, Wnt signaling, examples continue to accumulate in which Wnts and/or other key components of the canonical signaling cascade participate in β-catenin-independent processes.

5.3.1 Wnt signaling through β-catenin

The defining event in canonical Wnt signaling is the cytoplasmatic accumulation of β-catenin and its subsequent nuclear translocation and activity.

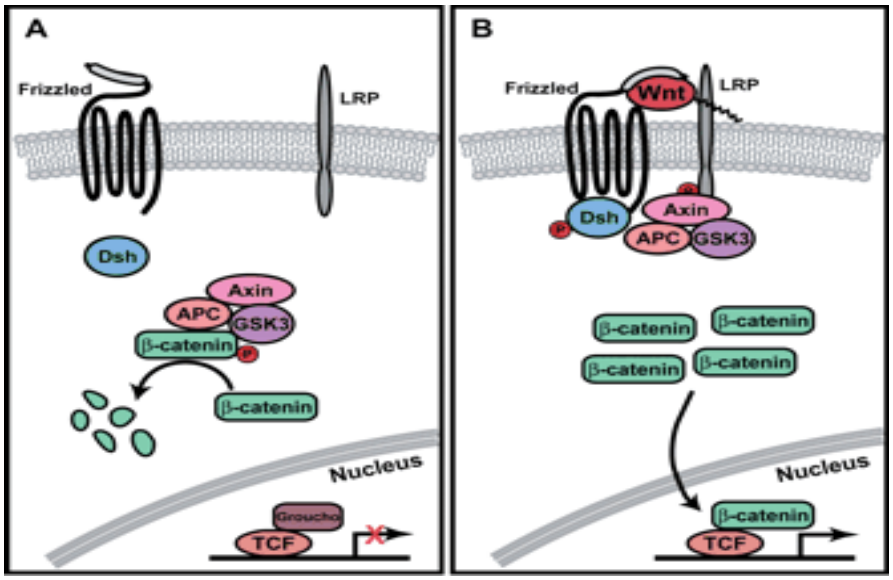

Figure 2. An overview of canonical Wnt signaling. *A*, in cells not exposed to Wnt, β-catenin associates with and is phosphorylated by the destruction complex composed of Axin, APC, and GSK-3. Phosphorylated β-catenin is then targeted for degradation. At the same time, Wnt target genes are repressed by the association of TCF with Groucho. *B*, Wnt binding to the Frizzled and LRP receptors induces phosphorylation of LRP and recruitment of Axin. Dsh is also phosphorylated, and the Axin.APC.GSK-3 complex is inhibited, leading to accumulation of cytosolic β-catenin. Accumulated β-catenin then translocates to the nucleus, replaces Groucho from TCF, and activates target genes. Adapted from [49]

Under unstimulated conditions, a β-catenin destruction complex formed by proteins that include Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3b (GSK-3) keeps cytoplasmatic levels of β-catenin low through phosphorylation by GSK-3. Phosphorylated β-catenin becomes ubiquitylated and is targeted for degradation by the proteasome [50]. Following Wnt binding to a receptor complex composed of members of the Frizzled (Fz) family of seven transmembrane, serpentine receptors and low density lipoprotein receptor-related protein (LRP), the Axin.APC.GSK-3 complex is inhibited, leading to a block in β-catenin phosphorylation by GSK-3. Hypophosphorylated β-catenin accumulates in the cytoplasm and is

translocated to the nucleus where it regulates target gene expression through partnerships with T cell specific transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF) family of transcription factors resulting in changes in gene transcription [51]. Remarkable progress has been made on this pathway, which has revealed the importance of Wnt–β-catenin signaling not only in metazoan development, but also in degenerative diseases and cancer. Whereas extensive study of Wnt–β-catenin signaling has resulted in its understanding in great (though incomplete) detail, noncanonical Wnt signaling has remained less well defined, with multiple β-catenin–independent pathways potentially existing side by side.

5.3.2 β-catenin independent Wnt signaling

The best-understood variant Wnt pathway was first described in *Drosophila*, where it was shown to be instrumental for the establishment of planar cell polarity (PCP), a process in which fields of cells orient themselves relative to the plane of the tissue in which they reside. However, whereas there are clear roles for *frizzled* (which was discovered because of its polarity phenotype) and *dishevelled* in this pathway, neither LRP, β-catenin, nor TCF are involved [52, 53].

Experiments performed largely in *Xenopus* and zebrafish embryonic systems have suggested the existence of a "Wnt-Ca^{2+}" signaling pathway [54, 55], in which the binding of Wnt promotes Frizzled-mediated activation of pertussis toxin-sensitive heterotrimeric guanine nucleotide–binding proteins (G proteins) [56]. This, in turn, stimulates the release of Ca^{2+} from intracellular stores, which leads to the activation of Ca^{2+}-dependent effector molecules. Several Ca2+-sensitive targets - protein kinase C (PKC), Ca2+-calmodulin–dependent protein kinase II (CamKII), and the Ca2+-calmodulin- sensitive protein phosphatase calcineurin - have been identified downstream of the Wnt-Ca2+ pathway in vertebrates [55]. Targets of the Wnt-Ca2+ pathway appear to cross-talk to the Wnt–β-catenin pathway at multiple points [57].

Because Wnts can interact with more than one Frizzled, detailed analysis of individual Frizzleds may yield new insights into Frizzled-specific pathways of signaling and gene expression. The role of low-density lipoprotein receptor–related proteins 5 and 6 (LRP5 and LRP6) as coreceptors with Frizzled in the Wnt– β-catenin pathway raises the question of a possible similar role for LRP proteins in Wnt-Ca2+ signaling.

5.3.3. Role of Wnt pathway in regulation of inflammation

Genomic studies have detected upregulation of Wnt5a in macrophages and dendritic cells exposed to pathogens [58, 59] and during differentiation of monocytes into dendritic cells after exposure to GM-colony stimulating factor (CSF) and interleukin-4 [60]. Together, this suggests that Wnt5a expression contributes to innate and adaptive immune responses. Support of this, upregulation of Wnt5a has been observed in pathological conditions involving inflammation such as rheumatoid arthritis [61] and in tumor associated macrophages [62]. One of the first direct indicators for the involvement of the Wnt pathway in inflammation and immunity was obtained from a study in Drosophila. Gordon and colleagues illustrated that the Drosophila Wnt protein family member WntD is upregulated in the fly via Toll/NF-κB signaling and is involved in the innate immune system [63]. A subsequent study in human mononuclear cells demonstrated upregulation of Wnt5a in response to microbial stimulation required Toll/NF-κB activation, indicating a similar mechanism in man [64]. Interestingly, this study also demonstrated that Wnt5a upregulates the microbially-induced IL-12 response of antigen-presenting cells and IFN-γ production by mycobacterial antigen-stimulated T-cells, illustrating a functional involvement in the antimicrobial defense. This was the first implication of the involvement of Wnt signaling in bridging innate and adaptive immunity to infections. In a recent study utilizing an in vitro model of inflammatory macrophage activation, additional support for a role of Wnt5a in sustained macrophage activation is provided [39]. Macrophages stimulated with interferon (INF)-γ and endotoxin (LPS) consistently upregulated Wnt5a via Toll-like receptor activation. Wnt5a in turn upregulated expression of the proinflammatory genes, IL-6, IL-1β, IL-8, and macrophage inflammatory protein-1β (MIP-1β). Attenuation of these effects by APC and the anti-inflammatory cytokine IL-10 imply an active role of Wnt5a in the inflammatory response. This study is the first to provide a direct demonstration of relevance to pathological inflammation by showing higher levels of Wnt5a in patients with severe sepsis than healthy subjects.

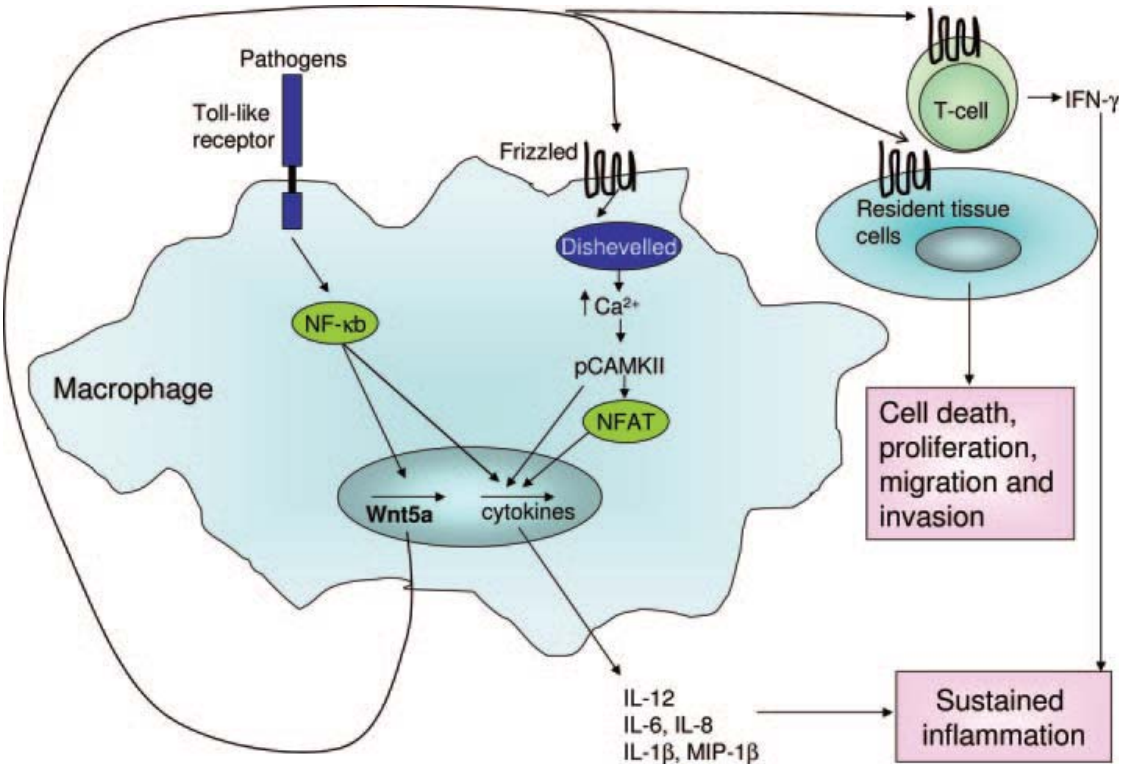

Figure 3. Working hypothesis of the role of Wnt5a in the inflammatory response. Activation of Toll-like receptors in macrophages by pathogens leads to activation of nuclear factor-κB (NF-κB) and upregulation of Wnt5a and cytokines. Increased levels of Wnt5a activate the noncanonical pathway via calcium/calmodulin dependent kinase II (CamKII) which results in sustained upregulation of inflammatory cytokines including IL12, IL6, IL8, IL1β, and macrophage inflammatory protein 1β (MIP-1β) either in a nuclear factor of activated T-cells (NFAT)-dependent or independent manner. Aditionally, increased levels of Wnt5a and cytokines may affect other mononuclear cells including T-cells and surrounding resident tissue cells. Adapted from [65].

5.4.Outline of the project

Macrophages play a central role in inflammation by responding to and releasing of numerous inflammatory cytokines and chemokines, leading to severe systemic inflammation and septic shock. However, the knowledge of anti-inflammatory interactions on the level of monocytes/macrophages is still scant. Therefore, it was the aim of this thesis to:

1. Define the transcriptional response of monocytes/macrophages to in vitro inflammation (LPS/INFγ).
2. Define new targets for the anti-inflammatory action of APC.
3. Elucidate the role of the Wnt pathway in inflammation.

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6. Publications and Submissions

6.1. Analysis of the Inflammatory Transcriptome of Human Macrophages Revealed Unique Genes as Targets for the Anti-inflammatory Action of Activated Protein C

Pereira C, Bachli EB, Schaer DJ, Schoedon G

Submitted 2008

6.2 Wnt5A/CaMKII signaling contributes to the inflammatory response of macrophages and is a target for the anti-inflammatory action of activated protein C and interleukin-10

Pereira C, Schaer DJ, Bachli EB, Kurrer MO, Schoedon G. Arterioscler Thromb Vasc Biol, 2008. 28(3): p. 504-510.

6.1. Analysis of the Inflammatory Transcriptome of Human Macrophages Revealed Unique Genes as Targets for the Anti-inflammatory Action of Activated Protein C

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Submitted 2008

Analysis of the Inflammatory Transcriptome of Human Macrophages Revealed Unique Genes as Targets for the Anti-inflammatory Action of Activated Protein C

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ABSTRACT

Activated protein C (APC) has been introduced as a therapeutic agent for treatment of patients with severe sepsis due to its unique anticoagulant and anti-inflammatory properties. In this study we investigated novel targets for the anti-inflammatory action of APC in inflamed macrophages.

Using a genome-wide approach, we profiled gene expression in human macrophages exposed to lipopolysaccharide (LPS) and interferon (IFN)-γ in the presence or absence of APC and identified for the first time genes regulated by APC under inflammatory conditions, such as chromatin binding protein 4B (CHMP4B), and p300/CBP-associated factor (PCAF), indicating a role of APC in epigenetic control of gene transcription. A functional assay showed the influence of APC in the acetyltransferase/deacetylase activity of nuclear extracts from inflamed macrophages.

Our data sheds new light on APC targets in inflammation and opens lines of investigation to be explored in order to further elucidate its unique molecule properties.

INTRODUCTION

The process of inflammation in response to injury or infection, a crucial part of the innate immunity, is characterised by activation, adhesion and transmigration of leukocytes through the vascular endothelium to the site of inflammation with the aim to locally repel microbial invasion and repair damaged tissues. In inflammation, immunovascular communication of circulating cells and vascular endothelium is mediated by production of pro-inflammatory factors such oxidation products, nitric oxide, inflammatory cytokines and chemokines, and the expression of adhesion molecules [1]. Although local inflammatory processes as defense mechanism are necessarily transient in nature, requiring a rapid induction and rapid termination, in severe infection they have significant potential to spread from the local site to non-affected tissues and elicit a systemic inflammatory response. Thus, severe systemic inflammatory response proceeds to a generalised intravascular inflammation with shock, organ dysfunction and high mortality [2]. Monocytes are an essential part of the innate immunity. They can differentiate into macrophages and dendritic cells and are crucial for the initiation of an adaptive immune response, clearance of infectious agents as well as resolution of inflammation. Inflammation due to tissue damage or infection results in macrophage activation, which increases the production of cytokines, chemokines, and other inflammatory mediators. The classical activation of macrophages is achieved by stimulation with interferon (INF)γ followed by exposure to a microbial trigger like lipopolysaccharide (LPS) resulting in a pro-inflammatory phenotype [1, 3]. By release of an array of pro-inflammatory mediators that communicate with cells of the vasculature they play a key role in the onset of systemic inflammatory responses. Because of the redundancy of inflammatory mediators involved, therapeutic intervention in severe systemic inflammatory response syndrome is challenging. Targeting the major inflammatory cytokines IL-1 or TNF-α, and lipopolysaccharide (LPS) was unsuccessful in treatment of sepsis. Furthermore, during severe systemic inflammation disseminated intravascular coagulation occurs because of the pro-adhesive endothelial environment and tissue factor expression on monocytes with consecutive intravascular thrombin formation [3, 4]. Activated protein C (APC), a plasma serine protease, is best known for its ability to control thrombin formation. APC acts as an anticoagulant by inhibiting activated clotting factors such as FVa and FVIIIa, thereby attenuating thrombin formation. In

addition to its anticoagulant properties, APC has been shown to modulate cell functions including inflammation, apoptosis, and vascular permeability [5]. In a previous study we found that APC affected the pro-adhesive endothelial environment by downregulating the induction of endothelial adhesion molecules and monocyte chemotactic protein-1 [6]. Recently, recombinant human APC has been introduced as a therapeutic agent for treatment of patients with severe sepsis due to its unique anticoagulant and anti-inflammatory properties [7]. However, the exact mechanism of the anti-inflammatory action of APC, in particular on inflammatory activated monocytes, is not fully understood.

Genome-wide approaches provide the ability to survey the expression level of thousands of genes simultaneously and are a powerful tools for exploring complex interactive networks of genes and signaling pathways. Because monocytes constitute an important cellular compartment involved in severe systemic inflammatory responses and to characterize novel targets for the anti-inflammatory action of the physiologic anticoagulant APC in this cellular system, we undertook expanded gene expression analysis of the whole human transcriptome.

In our present study we used a highly standardised setting of primary human monocytes activated with LPS and IFNγ and whole genome oligonucleotide arrays to define the complete transcriptome of blood monocytes in response to inflammatory activation. Moreover, we defined for the first time the gene expression profile of the effect of APC in inflammatory activated blood monocytes. Thereby we identified as targets of APC signalling molecules and transcription factors not known to be expressed in monocytes or assigned to inflammatory responses so far.

MATERIALS AND METHODS

Cell culture

Human macrophages were prepared from buffy coats of healthy blood donors (blood donation program; Swiss Red Cross, Zurich, Switzerland), by a strictly standardised protocol. Briefly, after separation by Ficoll gradient (Ficoll-paque^{TM} Plus, Amersham Biosciences, Amersham Basel, Switzerland) and three washes with Mg/Ca-free phosphate-buffered saline (PBS, Gibco Europe, Basel, Switzerland), cells were suspended in Iscove's modified Dulbecco's medium (IMDM; Invitrogen) supplemented with 10% heat-inactivated pooled human serum (human serum Off The Clott, PAA, Austria) and seeded at a density of 1 X 10^7 cells/mL in 6-well tissue culture plates (Falcon Oxnard, USA). Purified monocytes were obtained after 2h incubation under cell culture conditions in a SteriCult tissue culture incubator (Forma Scientific, Waltham, Massachusetts, USA) and four times washing in warmed Gey's balanced salt solution at a purity of > 98% as determined by Giemsa staining. After 24h, medium was replaced by IMDM supplemented with 2% human pooled serum. Cells were stimulated for 8h with human recombinant IFN-γ (100u/mL), IL-10 (5 ng/mL, both from PrepoTech, Rocky Hill, USA), lipopolysaccharide (LPS 10ng/mL, from Escherichia coli 055:B5, Difco Laboratories, Detroit, MI, USA), human recombinant activated protein C (rhAPC, Xigris^{TM}, Lilly, Switzerland).

DNA microarray hybridization and analysis

Differential gene expression profiling of human macrophages was performed as described previously [8].

Functional clustering

To analyze the microarray data in the context of biological functions we used information available from the Gene Ontology (GO) consortium (http://www.geneontology.org). The GO terms represent a defined vocabulary describing the biological process, cellular components, and molecular functions of genes in a hierarchical acyclic graph structure. Statistical analysis was performed using GeneGO software. For each of the existing GO terms, the cumulative number of genes meeting our criteria (e.g. up- or downregulated) and of all genes represented in the microarray was calculated. The Z score is calculated for every GO term as described [9]. A positive Z score indicates that there are more genes meeting

the criterion in the specific GO term than expected by chance. The Z score is transferred to p-values under the assumption of a hypergeometric distribution.

RNA isolation and quantitative real-time reverse transcription PCR (RT-PCR)

Total cellular RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Basel, Switzerland), which included a DNase digest. Total RNA was quantified spectrophotometrically and equal amounts (5 µg) were transcribed into cDNA with oligo(d)T primers and StrataScript RT Reverse Transcriptase using the StrataScript First-Strand Synthesis System (Stratagene, Rotkreuz, Switzerland). Duplicates of cDNA were amplified by RT-PCR with gene-specific primers using the 7500 Fast Real-Time PCR system (Applied Biosystems Inc., Rotkreuz, Switzerland) and the Power SYBR Green Master Mix (Applied Biosystems). Sequence-specific primers were selected using Primer Express v2.0 software (Applied Biosystems). The following primers were employed for Wnt5A and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): GAPDH forward, 5'-AAC AGC GAC ACC CAC TCC TC-3'; GAPDH reverse, 5'-GGA GGG GAG ATT CAG TGT GGT-3'. Primers for cytokines were as follows: IL1β-forward, 5’-CAG AAA ACA TGC CCG T-3’, IL1β reverse, 5’GCA CTA CCC TAA GGC AG-3’, IL8 forward, 5’-AGA CAG CAG AGC ACA CAA GC-3, IL8 reverse, 5’-ATG GTT CCT TCC GGT GGT-3’, MCP-1 forward, 5’-CCC CAG TCA CCT GCT GTT AT-3’, MCP-1 reverse, 5’-AGA TCT CCT TGG CCA CAA TG-3’, MIP1b forward, 5’-GAA AAC CTC TTT GCC ACC AA-3’, MIP1b reverse, 5’-TCA CTG GGA TCA GCA CAG AC-3’, HOXC10 forward, 5’-GAC CTG TGG TTC GTG C-3’, HOXC10 reverse, 5’-GCG GAT GGA TTC GAT CT-3’, TIGD4 forward, 5’-AGC AAC GAA GAG TGG T-3’, TIGD4 reverse, 5’-CTC TGG GGT TAC AGC C-3’, PCAF forward, 5’-GCA AGT CAA GGG CTA TGG-3’, PCAF reverse, 5’-GTG TAC GGG ATC CGT G-3’, CHMP4B forward, 5’-ACT TGT ACG GTA CTG GC-3’, CHMP4B reverse, 5’-TCG AGA TAT TTA ATA GAC AGT GC-3’, SOCS7 forward, 5’-CCG AAA GTT CTA CTA CTA TGA T-3’and SOCS7 reverse, 5’-AGA GTA CGG TCA TGT GC-3’. PCR was carried out with an initial denaturation step (10 min, 95°C) followed by 40 cycles of denaturation (15 sec, 95°C), annealing (30 sec, 55°C), and extension (30 sec, 72°C). Fluorescence was measured at the end of each extension. Relative mRNA levels were quantified by RQ Study SDS Software v1.3.1 (Applied Biosystems) using the comparative Ct method. The expression level of each gene was normalized to GAPDH levels in each experimental sample. Final data were

expressed as mRNA expression in treated cells relative to expression in untreated cells. A melting curve analysis was performed for each amplicon to verify the specificity of each amplification step.

Western blotting

For assaying HOXC10 and SOCS7 protein expression, cells were lysed with Mammalian Cell Lysis/Extraction reagent (Sigma-Aldrich Chemical Co., Buchs, Switzerland) supplemented with complete mini protease inhibitor cocktail tablets (Roche Diagnostics Schweiz AG, Rotkreuz, Switzerland). After clearing the lysates by high-speed centrifugation, protein concentrations of each sample were determined using a Protein Bradford assay (Bio-Rad Laboratories AG, Reinach, Switzerland). For immunoblotting, 20µg total protein of each sample was resolved on SDS 4–15% gradient polyacrylamide gels and transferred to PVDF membrane (Millipore AG, Zug, Switzerland). After transfer, the membranes were incubated for 1h in blocking solution (5% non-fat milk in PBS containing 0.1% Tween-20) and then 1h with mouse anti-HOXC10 (1:1000. abnova) or rabbit anti-SOCS7 (1:1000, abcam) in blocking solution. Antibody binding was detected with a horseradish peroxidase-coupled sheep-anti rabbit or donkey-anti rabbit secondary antibody diluted 1:10'000 followed by enhanced chemiluminescence (ECL) detection (ECL Plus, Amersham Pharmacia Biotech, Inc., Uppsala, Sweden).

Measurement of cytokine production

The inflammatory cytokines IL-8, IL-1β, MIP-1β and MCP-1 were measured in undiluted monocyte supernatants using a multiplex assay on the BioPlex 2200 platform (Bio-Rad, Hercules, CA, USA), with commercial antibody-coated beads, standards, and reagents, according to the manufacturer's instructions (Bioplex human cytokine multiplex assay system, BioRad). Complete medium was used as blank. Data were analyzed on the Bioplex Reader using the BioPlex 3.0 software (Bio-Rad).

Histone acetyltransferase (HAT) activity

HAT activity of nuclear extracts was measured with a colorimetric assay that measures NADH produced upon acetylation of a peptide substrate, following

manufacturer recommendations (HAT Activity Colorimetric Assay Kit, BioVision, California, USA).

Histone deacetylase (HDAC) activity

HDAC activity of nuclear extracts was measured with a colorimetric assay, following manufacturer recommendations (Colorimetric HDAC Activity Assay Kit, BioVision, California, USA).

RESULTS

Differential gene expression of inflamed human monocytes

Human monocytes prepared from buffy coats of healthy blood donors were cultured for 24h and then stimulated with INF-γ and LPS. Unstimulated monocytes were used as the baseline control. The transcriptional profiles were then determined by microarray analysis using Agilent human 44k 60-mer oligonucleotide microarray chips. Profiles of identical experimental settings from three independent donors were analysed with Rosetta biosoftware. After 8h stimulation, 2979 genes were regulated more than 2-fold ($p<0.01$) in these monocytes. Of the 2547 genes affected after 8h by INF/LPSγ stimulation, 1692 were upregulated, (with a maximal change of 222.5-fold) and 1287 were downregulated (with a maximal change of 18.0-fold). The maximally regulated genes were ankyrin repeat domain 22 (ANKRD22, 222-fold increased expression) and selenoprotein P (SEPP1, 18-fold decreased expression). The majority of differentially expressed genes were identified as unique and named in GenBank, whereas the remaining transcripts were either identified as unnamed expressed sequence tags or were hypothetical. The complete set of genes regulated upon inflammatory activation 2-fold or higher is deposited in the ArrayExpress database, accession number E-MEXP-927.

To determine which functional categories are overrepresented among regulated genes with a given statistical significance, we performed automated unbiased clustering using GeneGO software. Genes displaying at least 2.0-fold differential expression levels were classified into various categories based on the biological function(s) of the encoded protein to determine the global direction of the molecular response to inflammation (Figure 1). Classification according to GO biological processes revealed that most genes upregulated by LPS/INFγ fall into the functional

categories related to immune response and inflammatory response. In the downregulated genes, there was an overrepresentation of the protein kinase cascade and response to stress functional categories as well as an underrepresentation of categories related to metabolic processes.

Effect of activated protein C on inflamed macrophages

APC has been shown to down-regulate TNF production by monocytes and to inhibit the production of inflammatory cytokines and chemokines in LPS-stimulated THP-1 cells [10, 11].

To examine the effect of APC in our experimental setup, real-time PCR was used to verify the mRNA levels of IL-1β and IL-8 as well as MCP-1 and MIP-1β in all samples used for microarray experiments. As shown in Figure 2, APC downregulates the mRNA level of IL-1β, IL-8, MCP-1, and MIP-1β (Figure 2A) and the protein expression of MCP-1, and MIP-1β (Figure 2B) in monocytes stimulated with LPS/INFγ.

In order to define unknown targets of APC on inflamed macrophages, we compared the mRNA expression profiles of macrophages incubated with LPS/INFγ with or without APC. Re-ratio analysis using Rosetta Biosoftware follwed by one-way ANOVA [8], revealed 570 genes differentially expressed by inflammatory activation and these were significantly (more than 2-fold change, $p<0.05$) up- or down-regulated by APC. Of the 570 genes affected by APC upon LPS/INFγ stimulation, 440 genes were upregulated (with a maximal change of 14.8-fold), and 130 genes were downregulated (with a maximal change of 26.4-fold). The maximally regulated genes were tigger transposable element derived 4 (TIGD4, 14.8-fold increased expression) and angiomotin like 1 (AMOTL1, 26.4-fold decreased expression).

Among the genes downregulated by APC is Wnt5A, a gene that recently emerged to be an important factor for the Toll-mediated inflammatory signaling in macrophages. A detailed study of the anti-inflammatory impact of Wnt5A regulation by APC has recently been described [8].

To determine which functional clusters were overrepresented among APC regulated genes, we applied the same analysis (GeneGO software) as in the genes regulated by LPS/INFγ. Among the genes downregulated by APC, there was significant underrepresentation of genes involved in intracellular transport and protein transport and overrepresentation of genes involved in the adenylate inhibiting pathway and the

phospholipase C activating pathway. The genes upregulated by APC showed an overrepresentation of genes involved in the regulation of ATPase activity and an underrepresentation of genes involved in phosphoinositide metabolic process and negative regulation of protein amino acid phosphorylation.

Confirmation of microarray results by RT-PCR and Western Blot.

Using quantitative real-time RT-PCR we confirmed the expression patterns obtained by microarray analysis for selected genes related to regulation of gene transcription (TIGD4, HOXC10, CHMP4B and PCAF) and cytokine secretion (SOCS7) that have not been previously linked to the anti-inflammatory action of APC (Figure 4). The regulation was also confirmed at the protein level (HOXC10 and SOCS7). For the other gene transcripts (TIGD4, CHMP4B and PCAF) were no antibodies available. APC significantly upregulates TIGD4, PCAF and SOCS7 whereas HOXC10 and CHMP4B were significantly downregulated by APC.

APC alters the balance between acetylase and deacetylase activity in inflamed macrophages.

Transcription of eukaryotic genes is complex and depends on different coactivators, such as p300, PCAF and c-AMP response element-binding protein, as well as histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs are responsible mainly for destabilizing the chromatin structure to allow accessibility of different transcription factors. In contrast, HDACs serve as corepressors of gene transcription by restoring the condensation of DNA in chromatin.

Because PCAF was one of the genes significantly regulated by APC upon inflammatory stimulus and it has acetyltransferase activity, we hypothesized that APC might regulate activity of HATs. As shown in Figure 5A, APC significantly downregulate histone acetyltransferase activity in nuclear extracts of inflamed monocytes after 8h but not after 45min exposure. This indicates that APC has a long-term effect. As gene expression is regulated by a balance between HAT and HDACs, we also investigated APC effect on HDACs activity (Figure 5B) and found that APC inhibits HDAC activity in inflamed monocytes after 45min and 8h stimulation suggesting a fast and prolonged effect of APC on HDAC activity.

DISCUSSION

Circulating monocytes play a major role in the immediate host response to invading microorganisms. The primary functions of monocytes include the phagocytosis of invading pathogens and the synthesis and secretion of proinflammatory cytokines, chemokines and growth factors. In this study, we determined the transcriptional changes occurring in human monocytes after inflammatory stimulation (LPS and INFγ). The genes mostly regulated by inflammatory stimuli were ankyrin repeat domain 22 (increased expression) and selenoprotein P (decreased expression). The ankyrin-repeat domain (ARD) was first discovered as a repeated sequence in yeast cell-cycle regulation proteins [12, 13]. It is named after ankyrin, a cytoskeletal adapter protein, which contains 24 tandem copies of the repeat [13]. Since first being discovered, over 2800 ankyrin repeat proteins have been identified, each containing between three and 24 copies of the ankyrin repeat [14, 15]. Ankyrin repeats are common in signaling proteins, and appear to be general protein–protein interaction motifs [15]. The upregulation of ankyrin repeat domain 22 under inflammatory conditions is novel and may represent an increase of several proteins containing this domain that are involved in the inflammatory response, such as IκB [14]. Selenoprotein P is the major transporter of selenium, a trace element required for normal development [16]. The downregulation of selenoprotein P in our experimental setup is in line with previous findings that human and/or murine *Sepp1* gene promoter activity is impaired by IFN-γ, TNF-α, and IL-1β [17, 18] while induced by TGF-β [18] and IL-10 [19] suggesting a differential regulation of selenoprotein P expression during inflammatory reactions [14]. Functional clustering, of genes significantly regulated by inflammatory stimuli, showed a significant overrepresentation of genes involved in the inflammatory response (upregulated genes) and genes associated with lipid metabolic process (downregulated genes). The overrepresentation of downregulated genes associated with lipid metabolic process highlight the fact that macrophages are an active source of pro- and anti-inflammatory lipid mediators. Modulation of genes involved in general cellular metabolic activities is a prominent feature of macrophage differentiation and polarization. Also, macrophages are a major component of adipose tissue and play a role in the metabolic syndrome [20].

We and others have focused on the effects of activated protein C on monocytes, cells that play a critical role in initiating, perpetuating, and modulating the immediate host response to invading microorganisms. Activated protein C has been shown to exert direct anti-inflammatory effects on monocytes by inhibiting the production of proinflammatory cytokines [21, 22]. Using a genome wide approach we tried to identify novel targets for the anti-inflammatory action of APC. The genes most highly regulated by APC were TIGD4 (increased) and angiomotin like 1 (decreased).

TIGD4 belongs to the tigger subfamily of the pogo superfamily of DNA-mediated transposons in humans [23]. These proteins are related to DNA transposons found in fungi and nematodes. They are also very similar to the major mammalian centromere protein B [23]. Other novel APC targets were chromatin binding protein 4B (CHMP4B), and p300/CBP-associated factor (PCAF), which may indicate a role of APC in epigenetic control of gene transcription. In fact, PCAF has histone acetyltransferase activity, and through an acetyltransferase activity assay we were able to show that APC inhibits histone acetyltransferase activity in nuclear extracts from inflamed monocytes (Figure 5A). In health, the expression of genes coding for many proinflammatory cytokines remains silent. Mechanisms of transcriptional repression dominate until overcome by stimulation from extracellular stress signals such as microbial products via the Toll-like receptors. Although several transcription factors initiate de novo expression of proinflammatory cytokines, synthesis and secretion are tightly controlled events. Therefore, any mechanism for cytokine production is a potential target for acetylation and histone acetyltransferase (HAT) and deacetylase (HDAC) activity regulation. Acetylation of transcription factors can alter interactions between transcription factors and DNA and among different transcription factors, and is an integral part of transcription and differentiation processes. HATs are responsible mainly for destabilizing the chromatin structure to allow accessibility of different transcription factors, including NF-kB to the transcriptional site in the DNA. NF-kB can induce histone acetylation and other histone modifications in a temporal manner [24] leading to recruitment of other co-activator and remodelling complexes and the induction of inflammatory gene expression [25]. A recent study showed that HAT activity would be required to ensure the time-dependent induction of the inflammatory genes IL6, IL8 [26, 27], therefore an inhibition of HAT activity would contribute to the downregulation of these proinflammatory cytokines.

The inhibiton of HDAC activity by APC was unexpected, but several experimental studies have demonstrated that HDACs inhibitors can modulate immune responses [28-30]. Recent studies have shown that pretreatment with suberoylanilide hydroxamic acid (SAHA), an HDAC inhibitor significantly reduced LPS-induced secretion of TNF-α by peritoneal macrophages [28] by impairing transcription factor recruitment [31]. Our results suggest that APC exerts its anti-inflammatory action by altering the acetylation balance in inflamed macrophages.

Among the genes regulated by APC was also the homeobox domain C10 (HOXC10). HOXC10 is one of the highly conserved HOXC family members of transcription factors that play an important role in morphogenesis, cell differentiation, and proliferation [32-34]. The HOXC protein levels are controlled during cell differentiation and proliferation. Selected Hox proteins have been shown to directly and indirectly regulate the expression of many angiogenic and growth factors, including basic fibroblast growth factor, vascular endothelial growth factor, IL8, and Ang2 [34]. The functional role of HOXC10 in inflammation is a question that needs to be clarified in further studies.

SOCS (suppressor of cytokine signaling) proteins are known to act as negative regulators of cytokine action via inhibition of JAK/STAT signaling [35]. The SOCS family consists of 8 proteins (cytokine-inducible SH2-containing protein [CIS] and SOCS1-SOCS7). There is very little known about the function of SOCS7. It has been shown to interact with vinexin [36]. As vinexin is involved in the signal transduction from EGF-R to JNK and in cytoskeletal organization and cell spreading, SOCS7 could modulate these functions and thus play a role in adhesion-dependent signaling and in cytoskeletal remodeling in normal and transformed cells [37]. The observation that SOCS7 expression is induced by APC is novel and precise consequences resulting from this induction should be further elicidated.

FIGURE 1

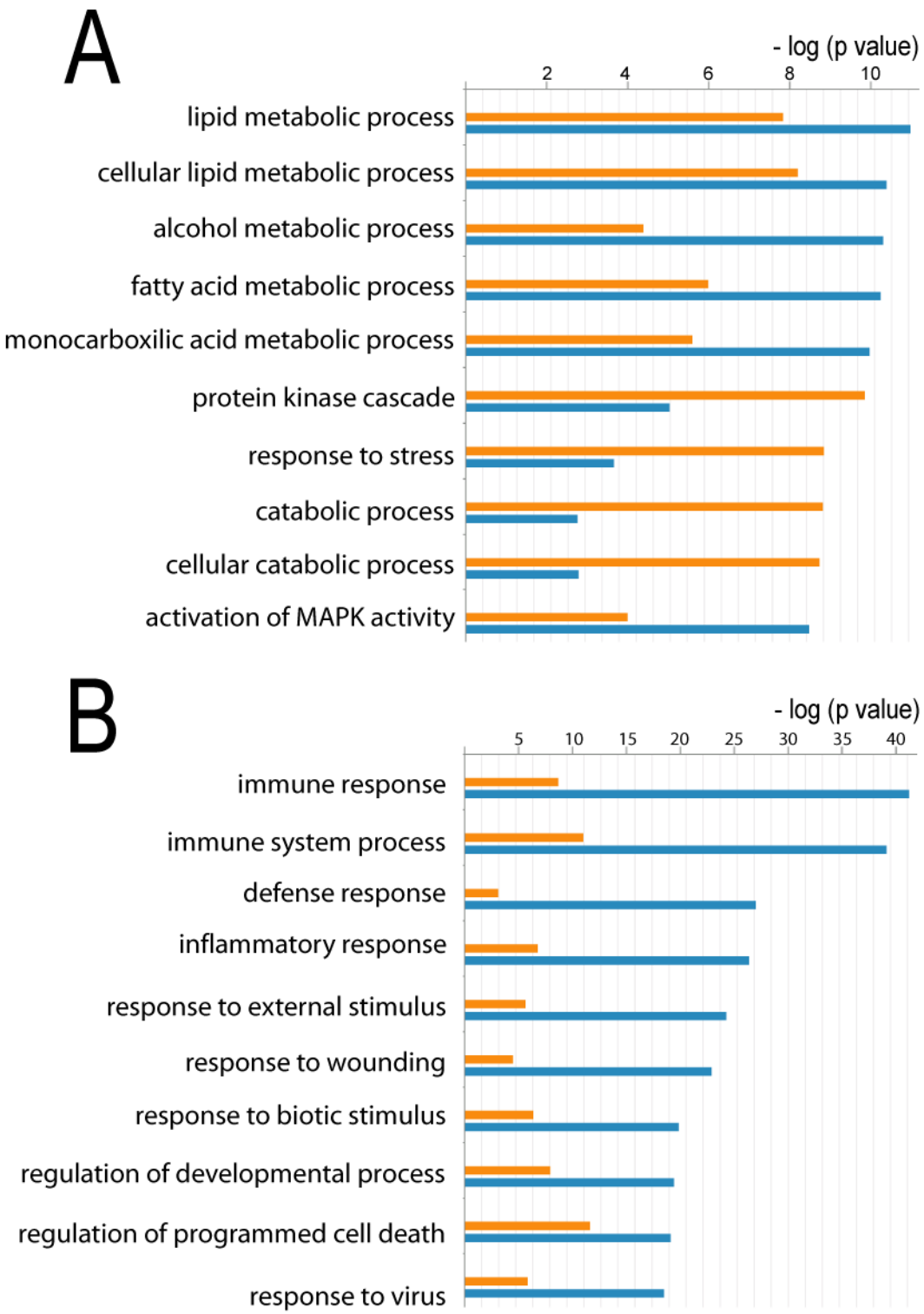

A
- log (p value)
2
4
6
8
10
lipid metabolic process
cellular lipid metabolic process
alcohol metabolic process
fatty acid metabolic process
monocarboxilic acid metabolic process
protein kinase cascade
response to stress
catabolic process
cellular catabolic process
activation of MAPK activity
B
- log (p value)
5
10
15
20
25
30
35
40
immune response
immune system process
defense response
inflammatory response
response to external stimulus
response to wounding
response to biotic stimulus
regulation of developmental process
regulation of programmed cell death
response to virus

FIGURE 2

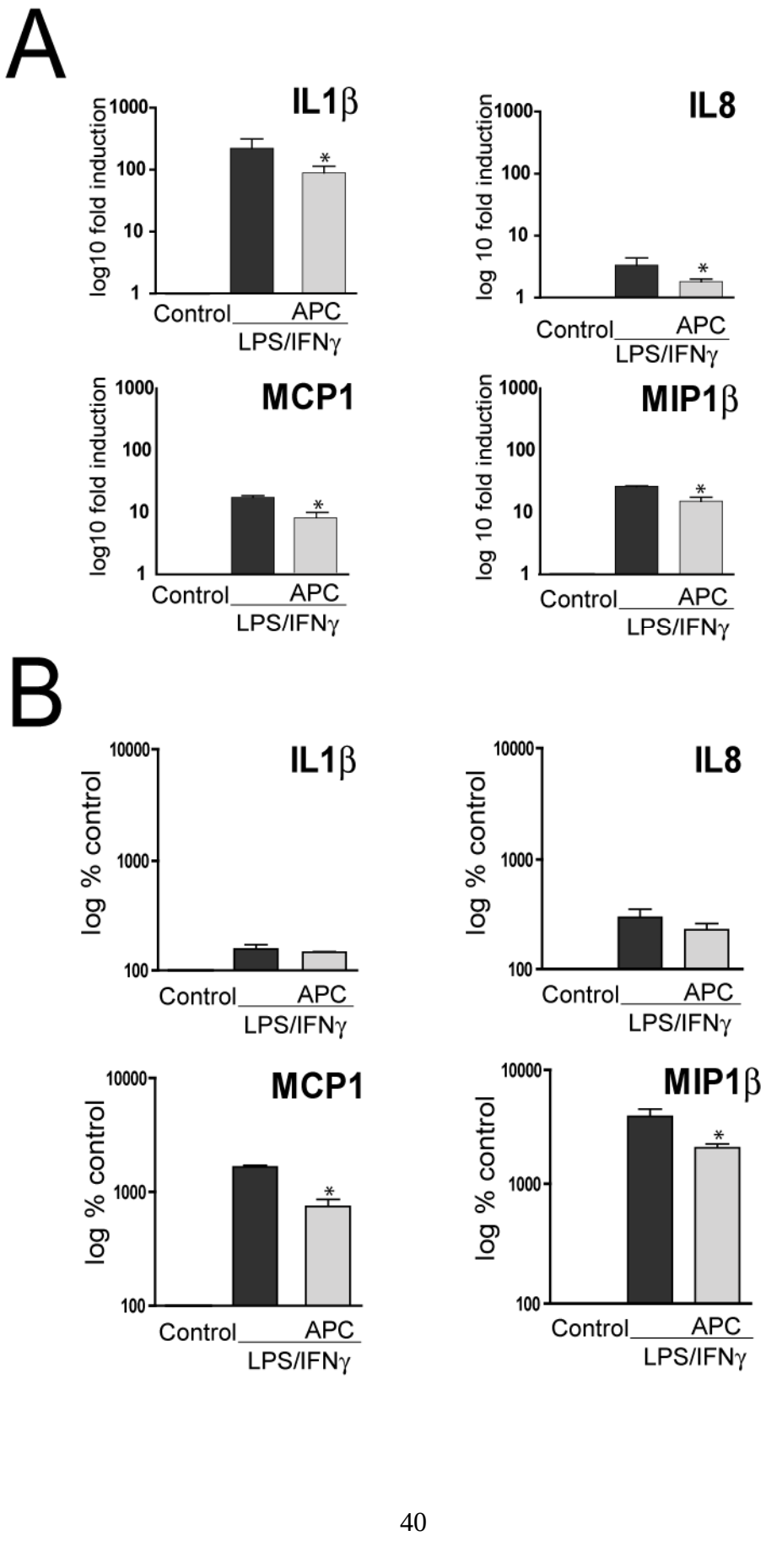
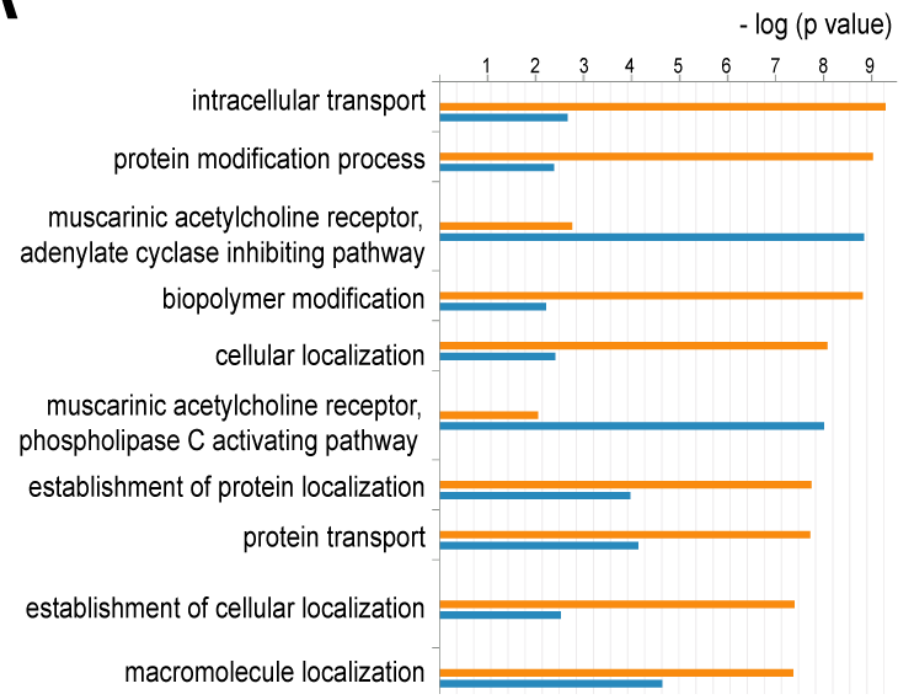

A
IL1β
log10 fold induction
1000
100
10
1
*
Control
APC
LPS/IFNγ
IL8
log 10 fold induction
MCP1
MIP1β
B
log % control
10000
1000
100

FIGURE 3

A


- log (p value)
1
2
3
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7
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9
intracellular transport
protein modification process
muscarinic acetylcholine receptor, adenylate cyclase inhibiting pathway
biopolymer modification
cellular localization
muscarinic acetylcholine receptor, phospholipase C activating pathway
establishment of protein localization
protein transport
establishment of cellular localization
macromolecule localization

B

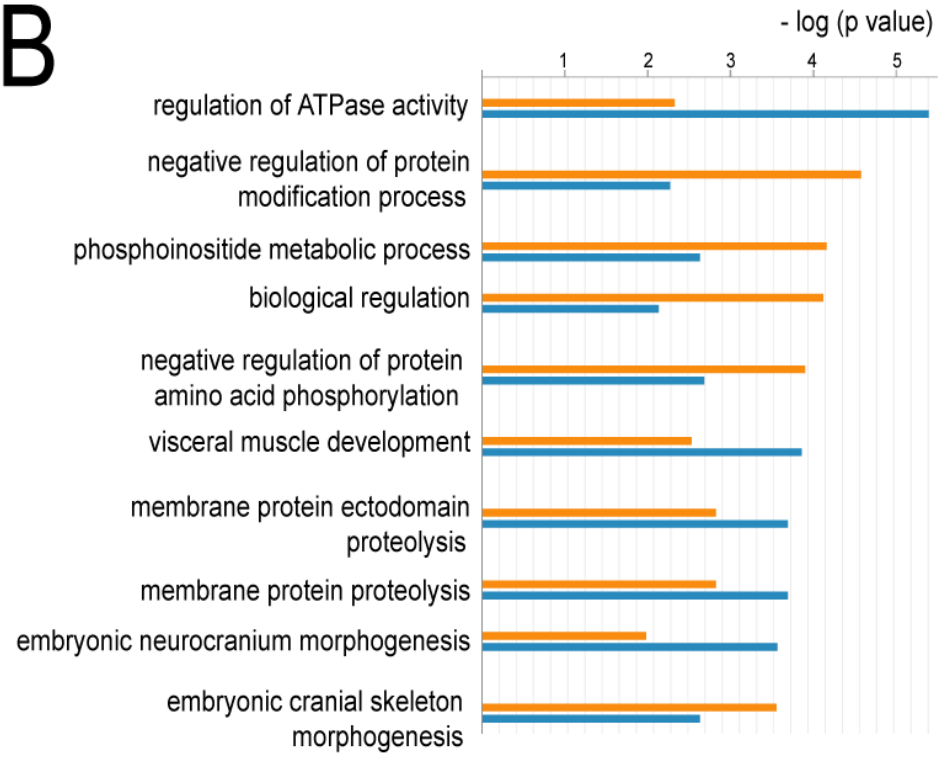

- log (p value)
1
2
3
4
5
regulation of ATPase activity
negative regulation of protein modification process
phosphoinositide metabolic process
biological regulation
negative regulation of protein amino acid phosphorylation
visceral muscle development
membrane protein ectodomain proteolysis
membrane protein proteolysis
embryonic neurocranium morphogenesis
embryonic cranial skeleton morphogenesis

FIGURE 4

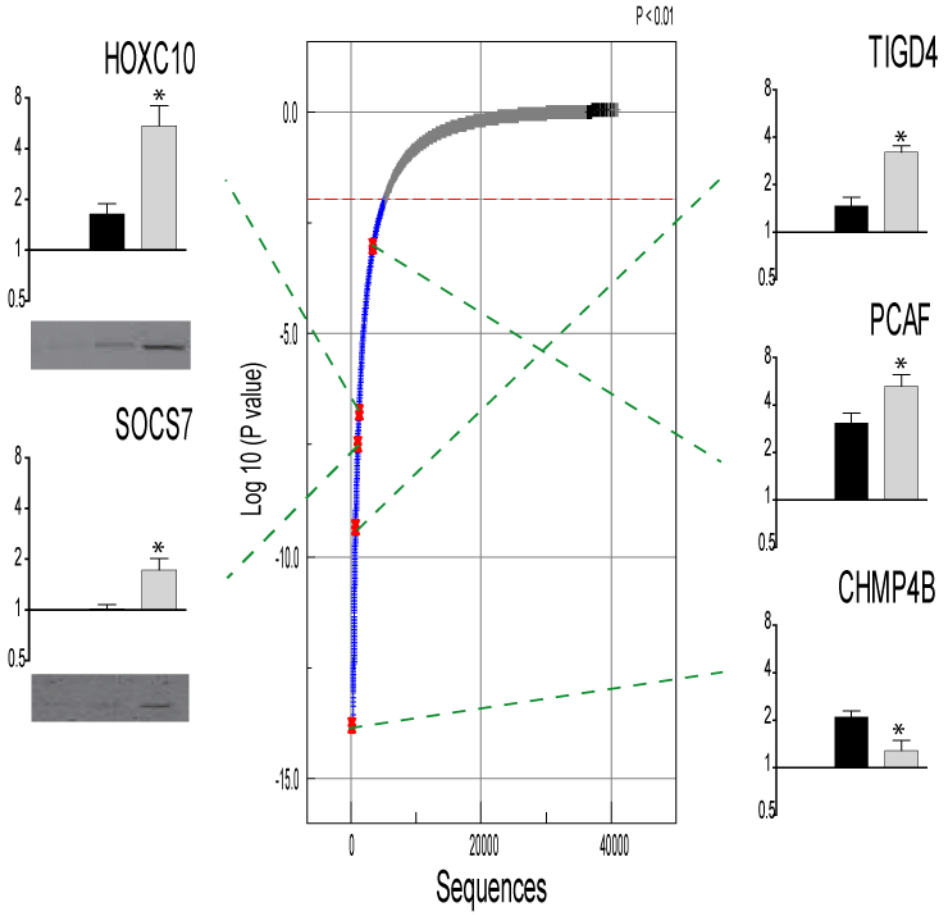
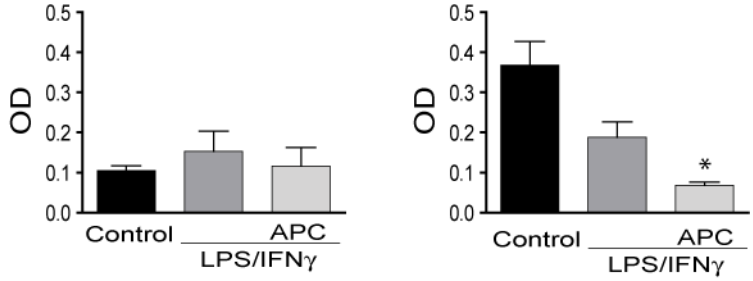

HOXC10
SOCS7
P<0.01
Log 10 (P value)
Sequences
0.0
-5.0
-10.0
-15.0
0
20000
40000
TIGD4
PCAF
CHMP4B

FIGURE 5

A


OD
0.5
0.4
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0.2
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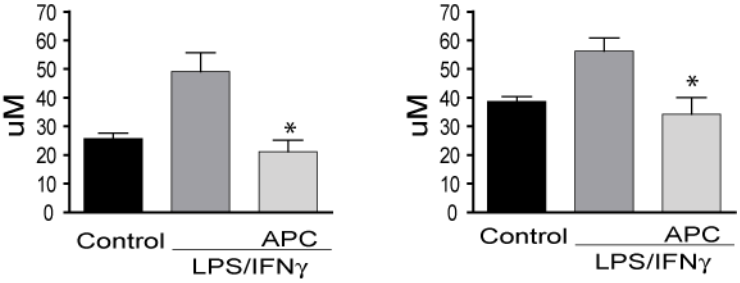
Control
APC
LPS/IFNγ
*

B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70
60
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40
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Control
APC
LPS/IFNγ
*

FIGURE LEGENDS

Figure 1 - Functional clustering of genes regulated by LPS/INFγ.

The gene expression profile of monocytes treated with LPS/INFγ compared with untreated control was analyzed with Rosetta Biosoftware [8]. Genes showing at least a 2.0 fold change were selected and characterized according to their GO biological process in the GeneGO software and compared with the classification of all genes present in the array in order to find which functional categories were significantly ($p<0.05$) over- or underrepresented [9] in the genes downregulated **(A)** or upregulated **(B)** by LPS/INFγ. The blue bars represent the genes regulated by LPS/INFγ and the orange bars represent all genes represented in the microarray.

Figure 2 – Regulation of inflammatory mediators by APC.

(A) Fold induction of IL1β, IL8, MCP1 and MIP1 β mRNA expression in LPS/INFγ stimulated monocytes treated with and without APC for 8h. Changes in mRNA expression were normalized to changes in GAPDH expression and are expressed as mean ± SD from at least three independent experiments. *$p<0.05$.

(B) Concentrations of IL1β, IL8, MCP1 and MIP1 β secreted by macrophages. Cells were stimulated with LPS/INFγ in the absence or presence of APC. Control cells were untreated. Cytokines were measured in cell culture supernatants collected 12h after treatment using the Bio-Plex Human Cytokine Multiplex Assay on the Bio-Plex 2200 platform. Cytokine concentrations in treated cells were normalized to the concentrations in control cells and are presented as mean ± SEM from three independent experiments. *$p<0.05$.

Figure 3 -Functional clustering of genes regulated by APC in inflamed macrophages.

The gene expression profile of monocytes treated with LPS/INFγ vs LPS/INFγ/APC was analyzed with Rosetta Biosoftware. Genes showing at least a 2.0 fold change were selected and characterized according to their GO biological process in the GeneGO software and compared with the classification of all genes present in the array in order to find which functional categories were significantly ($p<0.05$) over or underrepresented in the genes downregulated **(A)** or upregulated **(B)** by APC. The blue bars represent the genes regulated by LPS/INFγ and the orange bars represent all genes present in the array.

Figure 4 – Statistical analysis of APC effect on inflamed monocytes gene expression. In a first step of data analysis, Fold changes (up or down regulated) of gene expression were calculated using the re-ratio function of Rosetta Biosoftware that allows direct comparison between two samples (LPS/INFγ and LPS/INFγ/APC) that were both hybridized against a common reference (untreated control). All sequences with a calculated fold change of more than 2.0 ($p<0.01$) were subjected to one-way anova factorial analysis, compared with the significantly regulated sequences (fold change >2, *p* value<0.01) by LPS/INFγ. The ANOVA plot displays each gene (dot) in relation to its *p*-value (y axe) and the ranked sequence number (x-axe). Effects are considered significant at a *p*-value <0.01. Significantly regulated genes are plotted bellow the dashed red line. Highlighted genes, represented in red, were confirmed by real-time PCR (bar graphs, data are represented as mean ± SEM from three independent experiments. **p*<0.05) and when possible (antibody availability) by Western Blot.

Figure 5 – APC alters the histone acetyltransferase/deacetylase activity balance in inflamed monocytes. (A) Colorimetric determination of HAT activity in nuclear extracts of macrophages treated with LPS/INFγ with and without APC for 45min (left) or 8h (right). Data are represented as mean ± SEM from three independent experiments. **p*<0.05. (B) Colorimetric determination of HDAC activity in nuclear extracts of monocytes treated with LPS/INFγ with and without APC for 45min (left) or 8h (right). Data are represented as mean ± SEM from three independent experiments. **p*<0.05. (C) Chemiluminescent determination of MEF2 activity in nuclear extracts of macrophages treated with LPS/INFγ with and without APC for 45min. Data are represented as mean ± SEM from three independent experiments. **p*<0.05

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6.2 Wnt5A/CaMKII signaling contributes to the inflammatory response of macrophages and is a target for the anti-inflammatory action of activated protein C and interleukin-10

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Wnt5A/CaMKII signaling contributes to the inflammatory response of macrophages and is a target for the anti-inflammatory action of activated protein C and interleukin-10

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Running title: Wnt5A signaling in inflamed macrophages

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ABSTRACT

Objective-Sepsis is a major cause of death for intensive care patients. High concentrations of inflammatory cytokines are characteristic of severe systemic inflammation and activated monocytes are their predominant cellular source. To identify targets for anti-inflammatory intervention, we investigated the response of human macrophages to inflammatory and anti-inflammatory mediators.

Methods and Results- We profiled gene expression in human macrophages exposed to lipopolysaccharide (LPS) and interferon (IFN)-γ in the presence or absence of recombinant activated protein C (APC) or interleukin (IL)-10 and identified Wnt5A as one of the transcripts most highly induced by LPS/IFN-γ and suppressed by APC and IL-10. We confirmed regulation of Wnt5A protein in macrophages and detected it in sera and bone marrow macrophages of patients with severe sepsis. We established that a functional Wnt5A/frizzled-5/CaMKII signaling pathway was essential for macrophage inflammatory activation. To prove the essential contribution of Wnt5A we measured inflammatory cytokines after stimulation with Wnt5A, silenced Wnt5A by siRNA, and blocked receptor binding with soluble Frizzled–related peptide-1 (sFRP1).

Conclusion- Wnt5A is critically involved in inflammatory macrophage signaling in sepsis and is a target for anti-inflammatory mediators like APC or antagonists like sFRP1.

CONDENSED ABSTRACT

Gene expression profiling in human macrophages exposed to lipopolysaccharide and interferon-γ in the presence or absence of recombinant activated protein C or interleukin-10 identified Wnt5A as one of the most prominently regulated transcripts by these mediators. Wnt5A is critically involved in inflammatory macrophage signalling in sepsis.

INTRODUCTION

Sepsis is a suspected or proven infection with a systemic inflammatory response. In severe sepsis, organ dysfunction also occurs and it is associated with a high mortality and morbidity. Severe sepsis still causes about 9.3% of all deaths in the USA[1,2].

During sepsis, the extent of plasma protein C depletion correlates with the severity of the outcome[3]. In animal studies[4] and clinical trials activated protein C (APC) prevented death from severe sepsis or septic shock[5]. While this beneficial effect of APC is mostly ascribed to its anticoagulant properties, anti-inflammatory effects of APC have also been proposed[6]. The direct modulation of inflammation by APC has recently been described in gene expression profiling studies with human endothelial cells[7,8]. Recently, recombinant human APC has been introduced as a therapeutic agent for treatment of patients with severe sepsis due to its unique anticoagulant and anti-inflammatory properties; however, the exact mechanism of anti-inflammatory action is still unknown[9].

Macrophages play a central role in inflammation by responding to and releasing of numerous inflammatory cytokines and chemokines, leading to severe systemic inflammation and septic shock. However, the knowledge of anti-inflammatory interactions on the level of monocytes/macrophages is scant. Therefore, we decided to expand our investigations on anti-inflammatory effects of APC on this cellular system. In the present study, we were using a whole genome expression analysis approach, to define novel targets of APC in an *in vitro* model of inflammatory macrophage activation. Using probes obtained from human macrophages stimulated by interferon-γ (IFN-γ) and endotoxin (LPS), we consistently found Wnt5A to be one of the genes induced by inflammatory stimuli and LPS which was blocked by APC at the transcriptional level.

Wnt5A is a member of the Wnt family of secreted signaling molecules, homologs of the Wingless proteins in *Drosophila* species[10]. Wnt proteins are involved in embryonic development, in differentiation of white blood cells during mammalian hematopoiesis, and in tumorigenesis[11]. The canonical Wnt signaling pathway controls target gene transcription via the central component β-catenin[12]. Recently, regulation of β-catenin dependent gene transcription has been elucidated[13], and in mammalian cell systems it predominantly involves Wnt3[11]. However, in our pro-inflammatory activated human macrophage cellular system, Wnt3 expression was extremely low (see supplemental material, Table II, available online at

http://atvb.ahajournals.org) and the canonical signaling pathway was not affected by either pro-inflammatory activation or by APC and IL-10. Recently, a member of the *Drosophila* Wnt protein family, WntD, has been linked to Toll/NF-κB signaling and shown to be involved in antibacterial defence against *Listeria monocytogenes* in a septic fly model[14]. Furthermore, in a recent study Wnt5A and its receptor Frizzled-5 (FZD5) are involved in regulation of the response to microbial stimulation in human mononuclear phagocytes[15].

Here we show that Wnt5A acts through Ca^{2+}/calmodulin dependent protein kinase (CaMKII) and that this pathway contributes to the inflammatory response of human macrophages. APC and IL-10 modulate Wnt5A/CaMKII signaling in an anti-inflammatory manner. The presence of high levels of Wnt5A in sera of patients with severe sepsis or septic shock and in activated macrophages from the bone marrow of septic shock patients suggests a critical role for Wnt5A in systemic inflammation and sepsis. Taken together, our study shows for the first time that, first, Wnt5A signaling is essential to the general inflammatory response of human macrophages and, second, APC acts anti-inflammatory in activated human macrophages by interfering with Wnt5A signaling.

METHODS

Cell Culture

Human PBMC derived macrophages were cultured as described in the supplement (available online at http://atvb.ahajournals.org).

Gene Array Experiments

Gene expression profiling was performed by competitive dual-color hybridization of complementary RNA probes on human 44K 60-meroligonucleotide microarray chips (Agilent Technologies, Palo Alto, CA, USA) as described in the supplement.

Quantitative Real Time RT-PCR

Real-Time PCR is described in the supplement.

Antibodies

The following antibodies were used for western blotting and immunofluorescence: goat-anti-Wnt5A (1:1000, R&D Systems, Minneapolis, USA), rabbit-anti-FZD5 (1:1000, Abcam, Cambridge, UK), rabbit-anti-CaMKII (1:1000, Abcam), rabbit-anti-active CaMKII (pT^{286}; 1:500, Promega, Wisconsin, USA).

Western blotting

Detection of FZD5 and CaMKII protein expression was performed by Western blotting and is described in the supplement.

Immunohistochemistry and Immunofluorescence

Details about immunohistochemistry and immunofluorescence experiments are given in the supplement. Fluorescent signal intensity was quantified using SigmaScan-Pro software (Systat-Software Inc., San Jose, CA, USA).

Quantitation of secreted cytokines

Measurement of secreted IL-6, IL-8, IL-1β and MIP-1β is described in the supplement.

Immunoprecipitation of Wnt5A in sera

Detection of Wnt5A in the sera of septic patients and healthy individuals was performed by immunoprecipitation as described in the supplement.

Generation of small interfering RNA (siRNA) and transfection of macrophages

Wnt5A siRNA silencing experiments were performed as described in the supplement.

Statistical analysis

Data were analyzed with the use of Graphpad-Prism version 4.0 statistical software. We used an unpaired 2-tailed student t test or, for comparison of data among groups, one-way ANOVA followed by the Newman–Keuls test. p values <0.05 were considered statistically significant.

RESULTS

Identification of targets for the anti-inflammatory action of IL-10 and APC by gene expression profiling

To define novel targets for anti-inflammatory intervention, preferably at an early stage of the inflammatory response, we applied microarray based comparative transcriptome analysis in our model of monocyte derived macrophages.

The mRNA expression profiles of macrophages incubated with LPS/INFγ with or without IL-10 or APC for 8 h were compared with the expression profiles of untreated macrophages (common reference, cultured in parallel) by competitive two-color hybridization on human whole genome oligonucleotide array chips. The cytokine IL-10, that has well described anti-inflammatory properties, was used to compare its effect with the suspected but not elucidated, anti-inflammatory action of APC in our setting of inflammatory activated macrophages. Analysis of experiments with macrophages from three different donors revealed a set of genes induced by LPS/INFγ (see supplemental material, Table I, available online at http://atvb.ahajournals.org). Complete data are available in the ArrayExpress database with the following accession number, E-MEXP-927. In addition to several known inflammatory genes such as IL1β (87-fold induction compared to untreated cells), IL6 (15-fold), IL8 (3-fold), CCL2 (17-fold) and CCL4 (14-fold), Wnt5A was strongly induced by LPS/INFγ (79-fold, see supplemental table I). Anti-inflammatory stimuli reduced Wnt5A induction 2.7-fold by APC and 4.2-fold by IL-10 (Supplemental Figure I, available online at http://atvb.ahajournals.org). Furthermore, the FZD5 and CaMKII genes, which encode proteins involved in Wnt signaling pathways, were also differentially expressed. Both the genes for the receptor FDZ5 and for the signaling enzyme CaMKII are not upregulated by LPS/INFγ in presence of APC or IL10.

IL-10 and APC regulate the Wnt5A/Ca^{2+} pathway at the mRNA expression and protein level.

Quantitative RT-PCR verified that the expression and regulation of Wnt5A mRNA was induced by LPS/INFγ in human macrophages and that the level of induction of Wnt5A mRNA was reduced by the action of IL-10 and APC (Figure 1a). Transcriptional expression of Wnt5A was maximal at 8h in activated macrophage and declined rapidly within 24-48h to normal levels compared to unstimulated cells (data not shown). To test whether Wnt5A is also induced by signals targeting Toll-like

receptors (TLR), we investigated a representative array of TLR agonists targeting TLR 1–9, and found that only PamCSK4 (TLR 1/2 agonist) and Imiquimod (TLR 7 agonist) did not induce Wnt5A mRNA transcription. All other TLR agonists induced Wnt5A expression more than 10-fold (Figure 1b). Immunocytochemistry was used to assess the levels of accumulated Wnt5A protein in activated macrophages (Figure 1c). After 24 h incubation with LPS/INFγ, Wnt5A protein expression had increased in macrophages compared with untreated control cells. In contrast, after treatment with LPS/INFγ/IL-10 or LPS/INFγ/APC Wnt5A protein expression was unchanged in macrophages compared to untreated control cells (see Figure 1d). This observation leads us to the assumption that Wnt5A expression is induced by LPS/INFγ and that this effect is suppressed by IL-10 and APC.

FZD5 and CaMKII are present and contribute to Wnt5A signaling in macrophages that can be blocked by a sFRP1

Western blot analysis showed FZD5 and CaMKII expression in macrophages, providing further evidence that these important components of the Wnt5A signaling pathway are present (Figure 2a). FZD5 protein was present as a single band with a molecular weight of 250 kDa. There were no detectable differences in the amount of protein in response to different treatments. CaMKII was present as a single band with a molecular weight of 60 kDa. Although it was not possible to establish whether the amount of CaMKII protein increased in macrophages stimulated with LPS/INFγ, CaMKII protein expression clearly decreased due to the action of APC. To address CaMKII activation by Wnt5A, we used a specific polyclonal antibody that recognizes only the phosphorylated form of CaMKII. Activation of CaMKII by Wnt5A and by LPS/INFγ was confirmed by immunofluorescence in cultured macrophages (Figure 2b and c). To corroborate our evidence for the contribution of Wnt5A to the inflammatory response of human macrophages, we blocked Wnt5A signaling at the ligand/receptor stage. sFRP1 is a member of the sFRP family that contains a cysteine-rich domain homologous to the putative Wnt binding site of Frizzled proteins and it can act as a soluble modulator of Wnt signaling by specifically binding to Wnt5A[21]. Incubating macrophages with sFRP1 in addition to LPS/INFγ or recombinant Wnt5A reduced CaMKII phosphorylation, indicating that the Wnt5A signaling pathway was activated by inflammatory stimuli and that sFRP1 blocked this activation by preventing binding of Wnt5A to its receptor, FZD5 (Figure 2b, c).

Wnt5A stimulates the release of pro-inflammatory cytokines in macrophages**, and IL-10 or APC block the inflammatory action of Wnt5A**

The biologic response of macrophages to an inflammatory stimulus is the synthesis and secretion of an array of inflammatory cytokines and chemokines. In a next series of experiments we wanted to know if Wnt5A itself could account for a comparable effect on inflammatory cytokine production as LPS/IFN-γ. To investigate the biologic response to Wnt5A we quantified the levels of the proinflammatory cytokines IL-6, IL-8, IL-1β and MIP-1β in supernatants of macrophage cultures treated for 24h with recombinant mouse Wnt5A. As shown in Fig. 3, recombinant Wnt5A, expressed in a mammalian cell system and without detectable endotoxin concentrations (see method section), did indeed stimulate the release of pro-inflammatory cytokines in macrophages (black bars) in a comparable manner to cells that were stimulated with LPS/INF-γ (data not shown). Furthermore, IL-10 (grey bars) or APC (open bars) prevented the inflammatory cytokine release upon stimulation with Wnt5A (Figure 3). There were again common and distinct effects of IL-10 and APC. The anti-inflammatory effects of IL-10 and APC were comparably strong reducing IL-1 and MIP-1 production, while the effect of APC on IL-6 or IL-8 production was not as impressive as IL-10.

Blocking Wnt5A signaling influences the expression of inflammatory cytokines

To examine the Wnt5A contribution to the inflammatory response in macrophages further, we generated small interfering RNA (siRNA) against Wnt5A by transcription and dicing of a human Wnt5A specific template and investigated whether inhibition by siRNA of Wnt5A transcription influences the transcription and secretion of inflammatory cytokines. Transfection of unstimulated macrophages with anti-Wnt5A siRNA decreased Wnt5A activity by 80%, but did not significantly affect macrophage viability (data not shown). However in LPS/INF-γ stimulated macrophages, knockdown of Wnt5A significantly decreased transcription of Wnt5A (Figure 4a). To ensure specificity of the observed effect of siRNA directed against Wnt5A, we measured expression of the housekeeping gene HPRT and found no significant change (Figure 4a). Moreover, knockdown of Wnt5A significantly decreased transcription and secretion of the inflammatory cytokines IL-1β, IL-6 and IL-8 (Figure 4b). Transfection with siRNA directed against luciferase GL3 duplex had no effect on the response to LPS/INFγ compared to mock-transfected cells (data not shown).

These results further support a mechanism by which Wnt5A influences the transcription and release of inflammatory cytokines. Because of our previous observation that sFRP1 blocked CaMKII phosphorylation induced by LPS/INFγ stimulation we incubated stimulated human macrophages with sFRP1. We verified that sFRP1 inhibited the release of IL-1β, IL-6, IL-8 and MIP-1β in macrophages cultured under inflammatory conditions, which confirms that Wnt5A signaling caused secretion of pro-inflammatory cytokines (Figure 4c).

Wnt5A protein is present in the serum and bone marrow of patients with severe sepsis or septic shock

Due to the high levels of Wnt5A induced by inflammatory stimuli *in vitro*, we determined whether Wnt5A is present *in vivo*. Because Wnt5A is secreted, we suggested its presence in sera of septic patients. Thus we performed immunoprecipitation in archive sera samples from patients with severe sepsis or septic shock defined according to the criteria set forth by the 2003 International Sepsis Definitions Conference[16] using a Wnt5A specific polyclonal antibody. Supernatants of transfected cells expressing and secreting large amounts of Wnt5A[16] served as controls (Figure 5a). Densitometric analysis showed a distinct difference between the amounts of Wnt5A protein in patient samples compared with sera from healthy individuals (Figure 5b). Wnt5A concentration in sera of patients could be affected by kidney failure or disturbances of liver function, which occur often in patients with severe sepsis or septic shock. Serum creatinine concentrations were normal in all sera tested. In addition, neopterin, a marker of activated human macrophages was significantly elevated only in the sera of sepsis patients (Figure 5b). The finding of elevated Wnt5A in sera of patients suggests an active role for secreted Wnt5A in the pathophysiology of sepsis. To confirm this observation, we demonstrated Wnt5A expression in macrophages of bone marrow samples of patients with fatal sepsis[17]. Immunohistochemical analysis showed that there was intense staining for Wnt5A in hemophagocytic macrophages in these patients compared with bone marrow from healthy individuals. Again, sections of Wnt5A expressing cells and L-cells served as positive and negative controls, respectively, as they were fixed and stained in the same manner. The intensity of Wnt5A staining in the untransfected L-cells (background staining) was comparable to that found in bone marrow sections of healthy individuals (Figure 5c).

DISCUSSION

In this study we found that expression of Wnt5A is pivotal in a pathway involved in sustained inflammatory macrophage activation during sepsis. Our initial studies aimed to identify novel targets of the anti-inflammatory activity of APC in pharmacological doses used in sepsis patients, in macrophages.

Beyond modulation of coagulation activation, the pleiotropic anti-inflammatory activities of APC that are directed to different cellular targets are proposed to contribute to the uniquely beneficial activity of this agent in patients with severe sepsis. In a genome wide transcriptional screen of macrophages stimulated with LPS and IFN-γ, we have identified Wnt5A as one of the most highly induced genes. Suppression of inflammation-forced Wnt5A expression by APC and IL-10 implicated Wnt5A in an active role in the inflammatory response. Both the inflammatory driven transcriptional expression of Wnt5A and its suppression by APC and IL-10 were confirmed by independent methods at the mRNA and protein levels. Furthermore, high levels of immunoprecipitatable Wnt5A in sera from patients with severe sepsis provided direct evidence of an active role for secreted Wnt5A in the pathophysiology of the systemic inflammatory response during sepsis. Although our studies did not intend to identify the cellular source of Wnt5A secretion during sepsis, demonstration of Wnt5A positive macrophages within the bone marrow of patients with fatal sepsis suggests that activated macrophages play an important role in this disease setting.

Recently a distinct role for Wnt family members in inflammation and immunity has been recognized. The *Drosophila* Wnt protein family member WntD regulates the innate immune response to infection in the fly[14]. In these studies, WntD expression is shown to be controlled by Toll signaling, a highly conserved pathway known to induce antimicrobial and inflammatory responses also in the human macrophage. Furthermore, WntD signaling in the fly was independent of the common beta-catenin pathway, as it is the case in the inflammatory human macrophage system described herein. Moreover, WntD was identified as the first secreted regulator of Toll signaling in the fly. This is in line with an earlier study that described Wnt5A being secreted by activated antigen presenting cells and by inflammatory synoviocytes from rheumatoid arthritis joints[19]. This was the first report of Wnt5A expression associated with an inflammation in humans. However, It became unclear in this study, how Wnt5A expression was linked with inflammatory cytokine production, because the signaling of Wnt5A through FFZD5 was not delineated. More recently, Wnt5A expression is

shown to be induced by mycobacterial cell wall components and endotoxin in human antigen presenting cells and is dependent on activation of the central inflammatory regulator NF-κB[15]. In this report it is clearly shown that Wnt5A expression required Toll-like receptor (TLR)2 and TLR4-dependent signaling. This finding is completely in line with the finding of TLR dependent WntD expression in the fly, indicating that these pathways are linked together and are highly conserved throughout species. However, it was not elucidated whether activation of other TLRs induce Wnt5A or other Wnt homologues[15]. Our study extends the idea that Wnt5A secretion constitutes a highly conserved response to inflammatory macrophage activation by demonstrating that Wnt5A mRNA is not only induced by LPS and the classical macrophage stimulator INFγ, but also by a representative array of TLR agonists targeting TLR 1–9.

After secretion, Wnt5A is proposed to act by ligation to its receptor FDZ5, which we and others have shown to be expressed in human macrophages[15]. It was however not clear which pathway of Wnt5A signaling was involved in macrophages in the setting of inflammatory activation. Different signaling pathways may be triggered subsequently by Wnt5A/FDZ5. The canonical signaling pathway acts by enhancing the intracellular level of β-catenin. This canonical pathway is the most intensively studied pathway of Wnt action in cell development and differentiation. We were unable to detect any evidence for canonical signaling in our inflammatory activated macrophages. The non-canonical Wnt signaling pathway elucidated in the present study induced a rise in intracellular calcium levels and activated CaMKII, which, among other effects, leads to activation and nuclear translocation of the transcription factor NF-AT[20]. Cell specific characteristics and the co-receptor environment in particular, determine which of these pathways are activated upon ligation of a frizzled receptor with its Wnt ligand. Ca^{2+} release and the associated phosphorylation of CamKII are extensively studied signaling events that are critically involved in macrophage activation[21, 22]. Our finding that recombinant Wnt5A increased phosphorylation of CamKII provides experimental evidence that Wnt5A is capable of activating the pro-inflammatory Ca^{2+}/CamKII pathways in macrophages. This effect is completely abrogated by sFRP1, a soluble Wnt binding protein that specifically binds to and impairs Wnt5A interaction with its cognate cell surface receptor[18]. This implies that a specific Wnt5A/FDZ5 interaction mediates the Wnt5A activity observed in our experiments and excludes nonspecific effects imparted by small molecular

contaminants such as endotoxin. Again, this is fully in line with the findings of others that FDZ proteins are receptors for Wnt and that Wnt signaling is modulated by the specific cysteine-rich domain of soluble frizzled related peptides.[18] Our finding, that sFRP1 also inhibited the LPS/IFN-γ induced CamKII phosphorylation, implies a causal link between Wnt5A secretion triggered by inflammation and LPS/IFN-γ induced macrophage activation. Again, this Wnt5A/FDZ5 mediated activation of the non-canonical signaling pathway may thus act as a positive regulatory mechanism that sustains and enhances inflammatory macrophage activation induced by exogenous pro-inflammatory agents. Our finding that Wnt5A activates the non-canonical signaling pathway in macrophages is compatible with its capacity to exert inflammatory effects in rheumatoid synoviocytes and in antigen presenting cells. Furthermore, treatment of macrophages with exogenous Wnt5A initiated secretion of inflammatory cytokines in our study. However, the subsequent observation that Wnt5A knockdown by siRNA significantly decreased the transcription and secretion of inflammatory cytokines and that inhibition of autocrine Wnt5A signaling by sFRP1 almost completely reversed LPS/INFγ induced cytokine secretion suggests that Wnt5A is not just another inflammatory macrophage activator but also a pivotal regulator of macrophage activation during inflammation. In light of the findings in the septic fly model, where Wnt is critically involved in the control of infection, the findings of induced Wnt5A upon mycobacterial infection of human monocytes, and the identification of Wnt5A in inflamed synoviocytes of rheumatoid arthirtis, our present findings suggest that Wnt5A is a highly specific autocrinous and paracrinous macrophage derived effector molecule triggering inflammation through a well defined pathway. Therefore, our results suggest that the Wnt5A pathway is an attractive candidate target for therapeutic intervention in inflammatory diseases such as sepsis or rheumatoid arthritis. First, significant amounts of Wnt5A are secreted in septic patients. One could well assume from this finding and from the observations of Wnt5A being released by synoviocytes[19] that Wnt5A is elevated also in the sera of rheumatoid arthritis patients. Second, interference with the Wnt5A signaling pathway acts at an early step in macrophage activation. The data from this study together with previous evidence[17] suggests that Wnt5A signaling constitutes a non-redundant activation pathway, which might be indispensable for sustained inflammatory macrophage activation. Third, we show that expression of Wnt5A constitutes a highly conserved response to macrophage activation triggered by a wide array of ligands

involved in initiation of systemic inflammation. This in turn implicates Wnt5A signaling in a final common pathway of macrophage activation. Fourth, the fact that Wnt5A is a secreted protein and that Wnt5A signaling involves classical receptor–ligand interactions raises the possibility of modulation of Wnt5A activity *in vivo*. Application of soluble Wnt5A neutralizing molecules, as applied in our work, thereby represents just one possibility for achieving *in vivo* suppression of inflammatory Wnt signaling. While our results suggest that the development of Wnt antagonists might be a valuable strategy for controlling inflammation at one of its critical checkpoints, partial Wnt control during sepsis may yet be achieved by treatment with APC, which acts by downregulation of Wnt5A expression and thus by modulating inflammatory Wnt activity.

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Disclosures

None

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FIGURE LEGENDS

Detailed information to each figure is given in the supplement (available as supplemental data)

Figure 1. Wnt5A expression is induced by LPS/INFγ and TLR agonists and is blocked by IL-10 and APC.

(**a and b**) Wnt5A mRNA of unstimulated (control) or macrophages treated with the indicated stimuli. (**c**) Immunofluorescence of Wnt5A protein in macrophages treated for 24 h with the indicated stimuli. (**d**) Quantitative fluorescence signal intensity of Wnt5A in control and activated macrophages.

Figure 2. The Wnt5A signaling pathway is active in macrophages and is blocked by sFRP1.

(**a**) Detection of CaMKII and FZD5 protein in lysates of cultured macrophages treated with LPS, INFγ, IL-10 and APC, as indicated, for 24 h. (**b**) Immunofluorescent detection of phosphorylated CaMKII. (**c**) Quantitative fluorescence signal intensity of phosphorylated CaMKII in control and treated macrophages.

Figure 3. Induction of inflammatory cytokine secretion by Wnt5A signaling, its modulation by anti-inflammatory mediators IL-10 and APC.

Concentrations of IL-6, IL-8, IL-1β and MIP-1β protein secreted by macrophages treated with Wnt5A in the absence and the presence of IL-10 or APC, respectively.

Figure 4. Effect of blocking Wnt5A signaling with siWnt5A on inflammatory cytokines secretion

(**a**) Left graph, fold induction of Wnt5A mRNA expression in LPS/INFγ stimulated macrophages after transfection with siWnt5A. Right graph, HPRT mRNA expression in LPS/INFγ stimulated macrophages after transfection with siWnt5A (**b**) IL-6, IL-8, IL-1β mRNA expression and secretion by LPS/INFγ stimulated macrophages after transfection with siWnt5A. (**c**) Concentrations of IL-6, IL-8, IL-1β and MIP-1β secreted by macrophages. Cells were stimulated with LPS/INFγ in the absence or presence of sFRP1.

Figure 5. Wnt5A protein is present in the serum and bone marrow of patients with severe sepsis or septic shock.

(**a**) Immunoprecipitation of Wnt5A in serum from patients with sepsis (lanes 5–8) and from healthy individuals (lanes 1–4). Lane 9 shows Wnt5A conditioned medium (Wnt5A-CM) that was immunoprecipitated following the same protocol. (**b**) Left graph, densitometric analysis of bands detected by immunoprecipitation. (**c**) Wnt5A in sections from bone marrow biopsies of patients with fatal sepsis and from healthy individuals[17] was detected by immunohistochemistry as described in Methods.

FIGURE 1

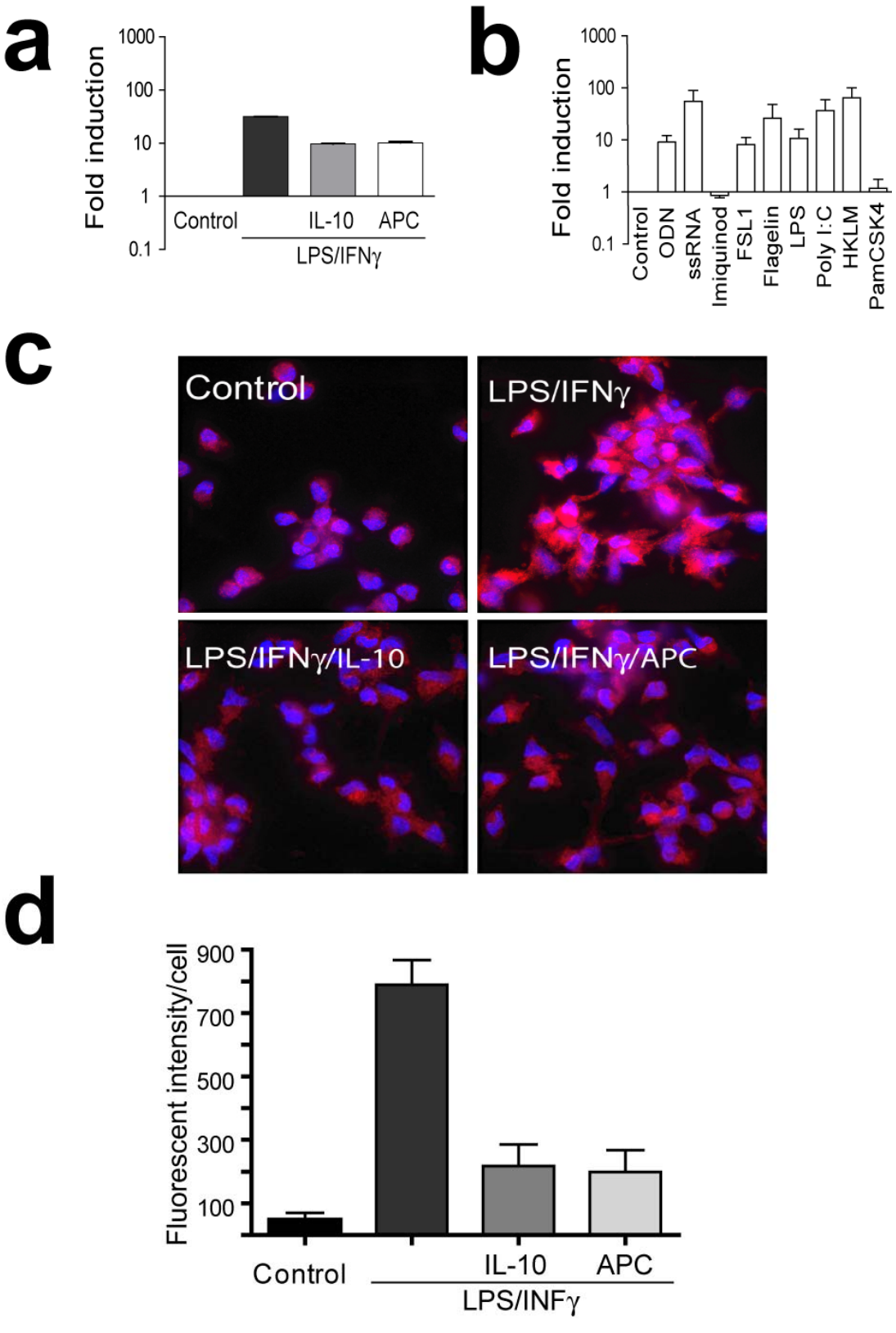
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FIGURE 2

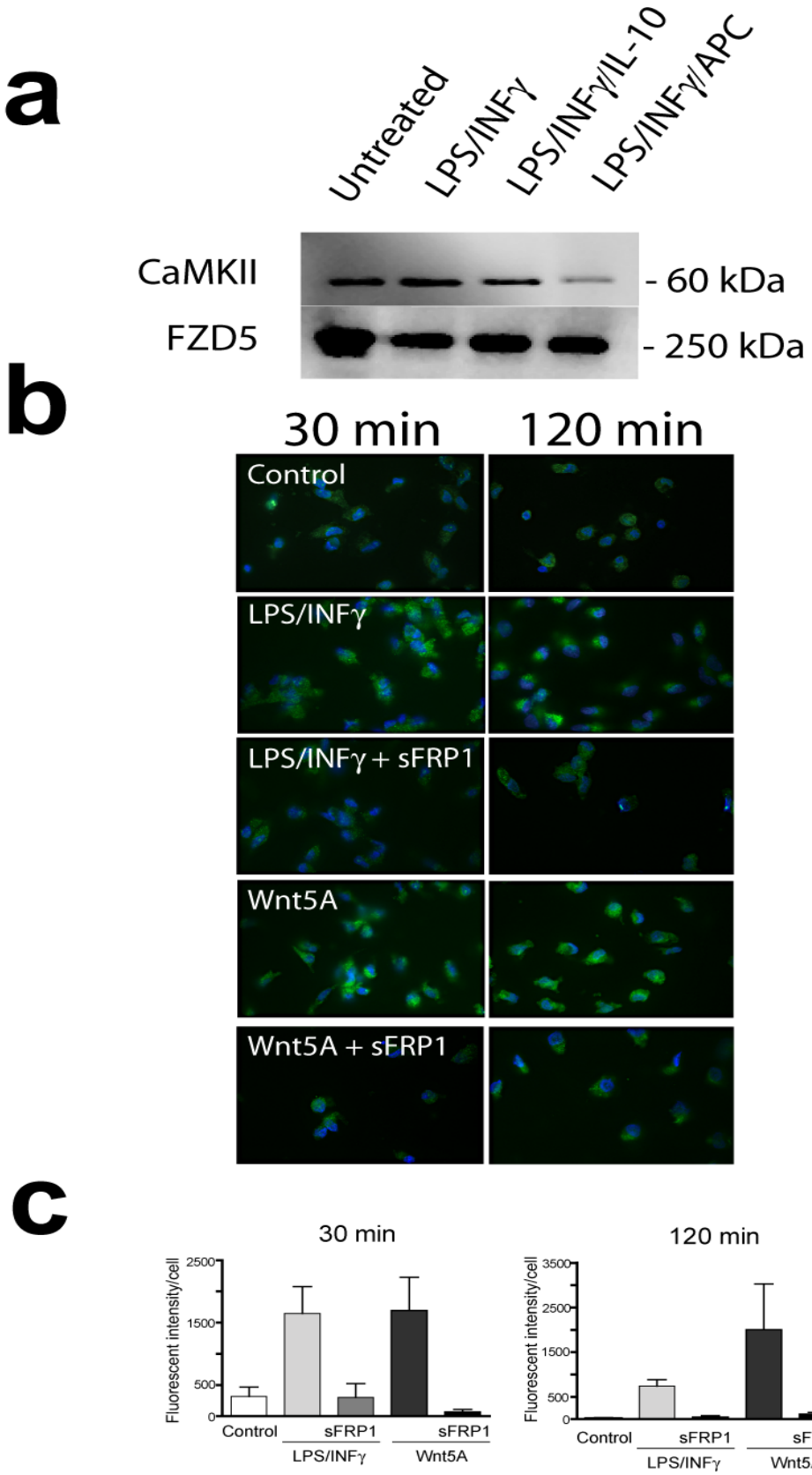
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FIGURE 3

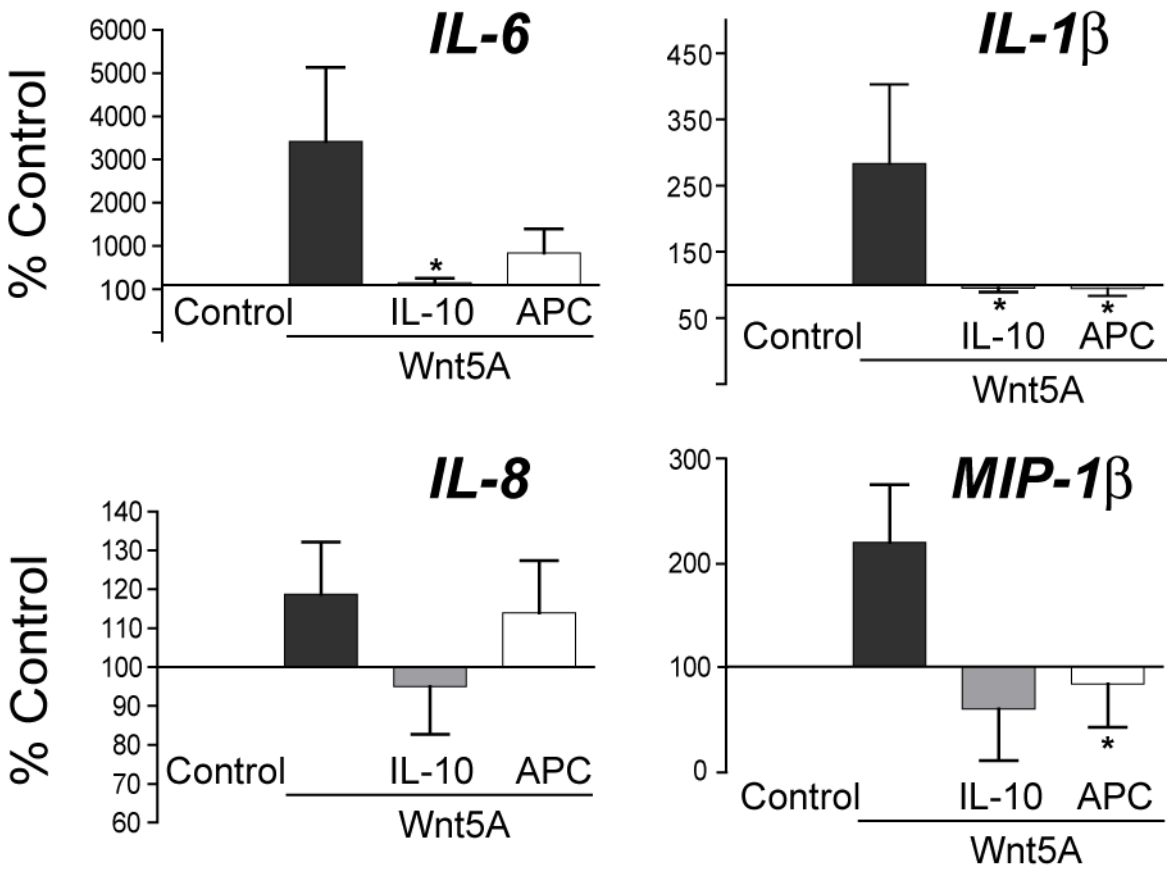
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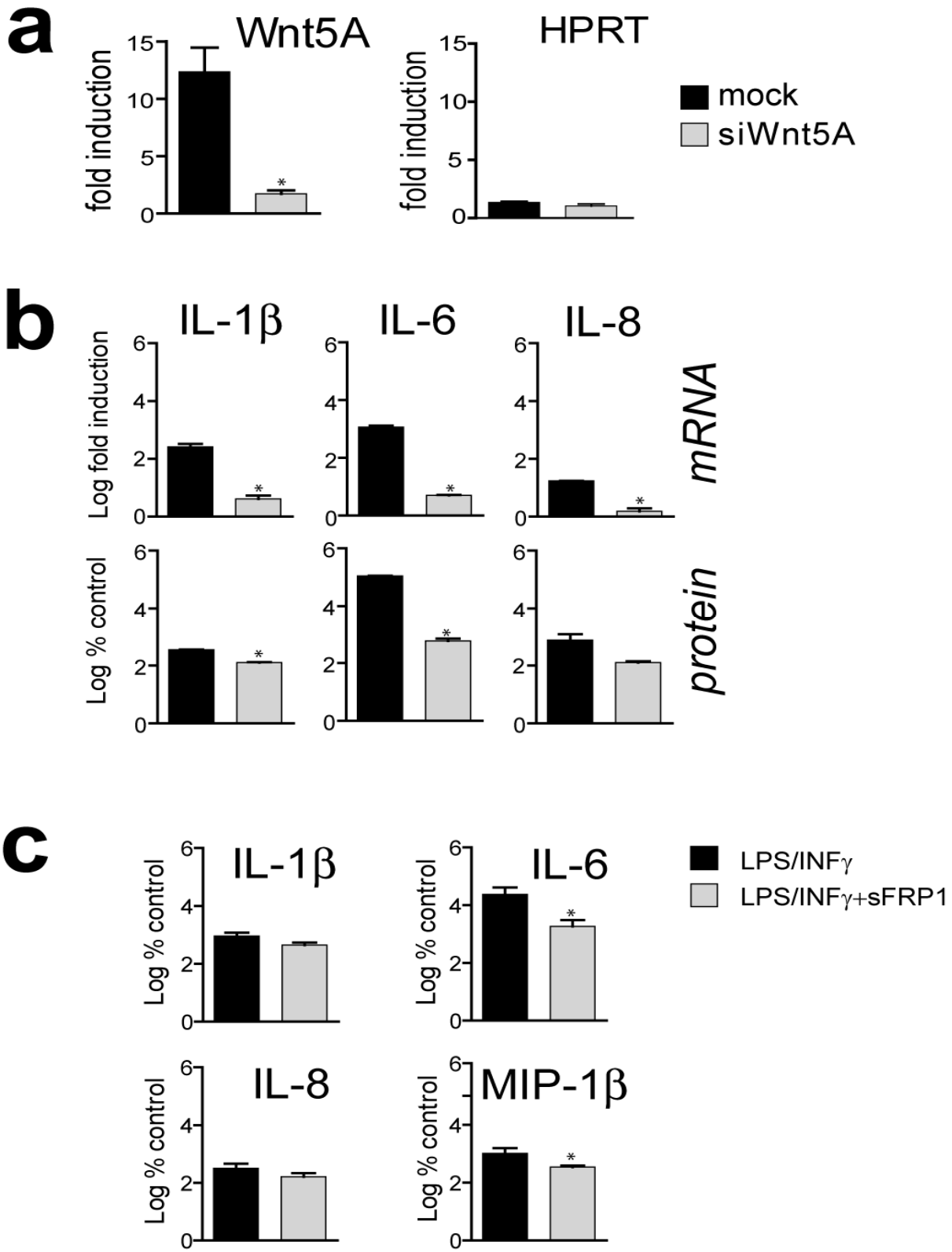
FIGURE 4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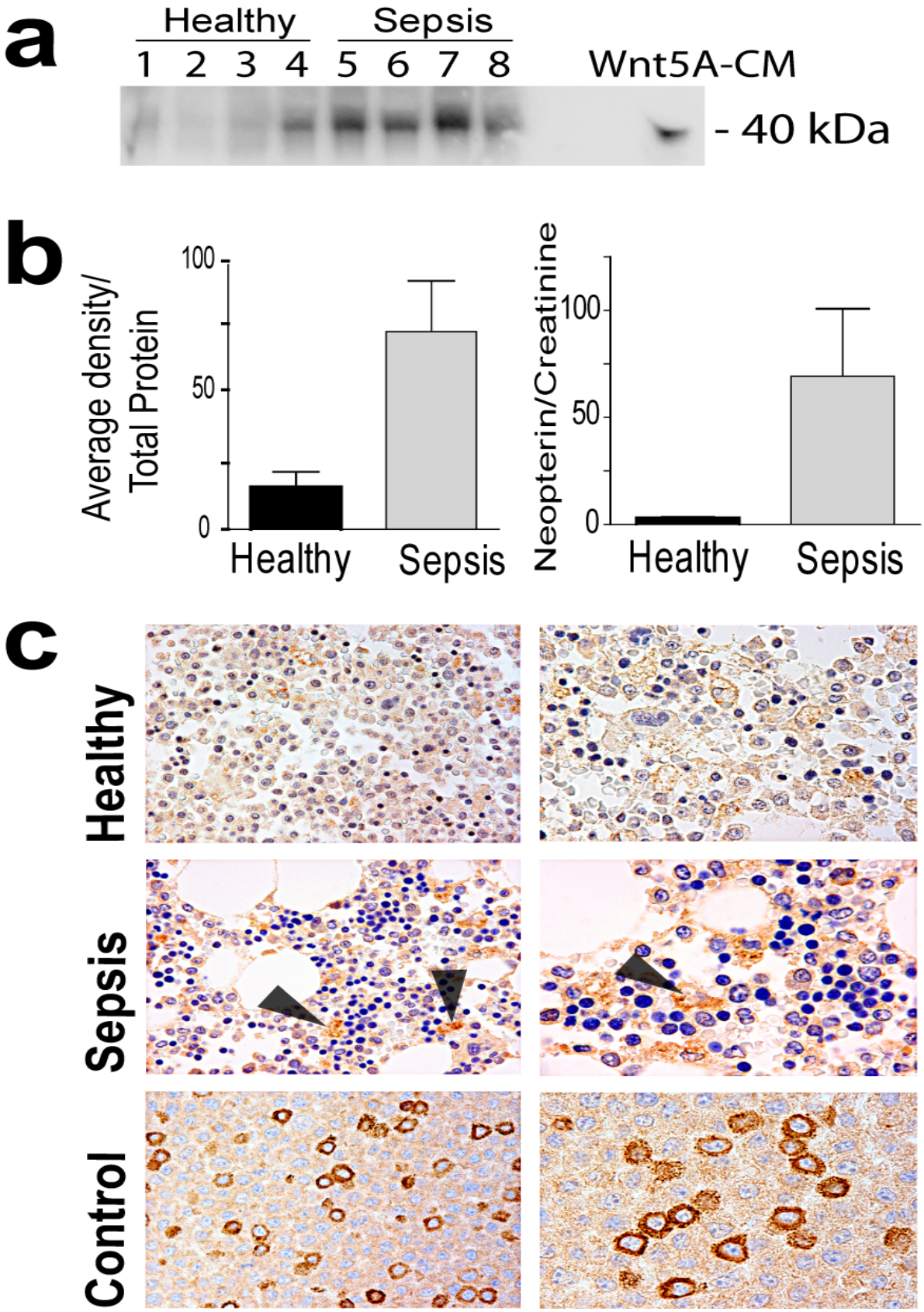
Wnt5A
HPRT
fold induction
15
10
5
0
*
mock
siWnt5A
b
IL-1β
IL-6
IL-8
Log fold induction
Log % control
6
4
2
0
*
mRNA
protein
c
IL-1β
IL-6
IL-8
MIP-1β
Log % control
LPS/INFγ
LPS/INFγ+sFRP1

FIGURE 5


a
Healthy
Sepsis
1 2 3 4 5 6 7 8
Wnt5A-CM
- 40 kDa
b
Average density/ Total Protein
100
50
0
Healthy
Sepsis
Neopterin/Creatinine
100
50
0
Healthy
Sepsis
c
Healthy
Sepsis
Control

SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Cell culture

Human macrophages were prepared from buffy coats of healthy blood donors (Swiss Red Cross, Zurich, Switzerland), by a strictly standardized protocol. Briefly, after separation by Ficoll gradient (Ficoll-Paque^{TM} PLUS, Amersham Biosciences) and three washes with Mg^{2+}/Ca^{2+}-free phosphate-buffered saline (PBS; Gibco Europe), cells were suspended in Iscove's modified Dulbecco's medium (IMDM; Invitrogen Europe) supplemented with 10% heat-inactivated pooled human serum (Human Serum, off the clot; PAA, Austria) and seeded at a density of 10^7 cells/mL in 6-well tissue culture plates (Falcon Oxnard, USA). Macrophages were obtained at a purity of >98% (determined by Giemsa staining) after 2 h incubation under standard cell culture conditions (37°C, 5% CO_2) in a SteriCult tissue culture incubator (Forma Scientific, USA). The cells were washed four times in warmed Gey's balanced salt solution and incubated for 24 h as before. Medium was then replaced by IMDM supplemented with 2% human pooled serum and cells were cultured for 48 h. For the last 8 h or 24 h cells received the following treatments prior to mRNA or protein expression analysis respectively: 100 U/mL human recombinant IFNγ, 5 ng/mL IL-10 (both from PeproTech, Rocky, Hill, USA); 10 ng/mL LPS (from *Escherichia coli* 055:B5; Difco Laboratories, Detroit, USA); 5 μg/mL human recombinant APC (Drotregocin alpha activated; Xigris^{TM}, Eli Lilly, Switzerland); 250 ng/mL recombinant mouse Wnt5A, 10 μg/mL recombinant human soluble frizzled-related peptide-1 (sFRP1: both expressed in chinese hamster ovary cells, no endotoxin detectable, from R&D Systems Europe Ltd., Abington, UK); and human TLR 1–9 agonist, 5 μM oligodeoxynucleotides (ODN), 0.5 μg/mL ssRNA40/LyoVec, 0.5 μg/mL Imiquimod, 500 ng/mL FSL1, 5 μg/mL Flagellin, 25 μg/mL Poly I:C, 10^8 cells/mL HKLM and 0.5 μg/mL PamCSK4 , 10 ng/mL *E.coli* K12 LPS (tlrl-kit, InvivoGen, USA).

DNA microarray hybridization and analysis

Differential gene expression profiling of human macrophages was performed by competitive dual-color hybridization of cRNA probes with untreated and treated cells cultured for 48 h on human 44k 60-mer oligonucleotide microarray chips (Agilent Technologies Schweiz AG, Basel, Switzerland). Total RNA was purified using the RNeasy Mini Kit protocol (Qiagen AG, Hombrechtikon, Switzerland) with on-column

DNase digestion according to the manufacturer's instructions. For quality control purposes and to quantify samples, each RNA sample (1 µL) was analyzed on RNA 6000 Nanochips using a Bioanalyzer 2100 instrument and 2100 Expert software (Agilent Technologies,). High quality total RNA typically had an 18/28S ribosomal RNA ratio >1.5 and was stored frozen in aliquots at –70°C. Labeled cRNA probes were synthesized from 1 µg total RNA using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) and cyanine-3-CTP or cyanine-5-CTP (Amersham Biosciences Europe GmbH, Freiburg, Germany). Labeled cRNA probes were purified on RNeasy Mini Spin Columns (Qiagen) and quantified in a NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Anaheim, USA). Equal quantities of Cy3- and Cy5-labeled probes (1 µg of each) were mixed, spiked with control targets and incubated in fragmentation buffer for 30 min at 60°C in the dark. After fragmentation, the gasket slides were completely filled with the respective probe mixtures. The hybridization chambers were assembled with Human 1A Oligo Microarray slides (Agilent Technologies) and hybridized for 17 h at 60°C in the dark with mixing at 4 rpm. After washing twice in SSPE/N-Lauroylsarcosine for 1 min, the slides were carefully washed in acetonitrile for exactly 30 sec and dried. Finally, slides were directly scanned using a dual-laser microarray scanner and analyzed with Feature Extraction software (Agilent Technologies). Data mining was achieved using the Rosetta Resolver Gene Expression Data Analysis System (Rosetta Biosoftware, Seattle, USA, www.rosettabio.com).

RNA isolation and quantitative real-time reverse transcription PCR (RT-PCR)

Total cellular RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Basel, Switzerland), which included a DNase digest. Total RNA was quantified spectrophotometrically and equal amounts (5 µg) were transcribed into cDNA with oligo(d)T primers and StrataScript RT Reverse Transcriptase using the StrataScript First-Strand Synthesis System (Stratagene, Rotkreuz, Switzerland). Duplicates of cDNA were amplified by RT-PCR with gene-specific primers using the 7500 Fast Real-Time PCR system (Applied Biosystems, Inc., Rotkreuz, Switzerland) and the Power SYBR Green Master Mix (Applied Biosystems). Sequence-specific primers were selected using Primer Express v2.0 software (Applied Biosystems). The following primers were employed for Wnt5A and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): Wnt5A forward, 5'-AGT TGC CTA CCC TAG C-3'; Wnt5A

reverse, 5'-GTG CCT TCG TGC CTA T-3'; GAPDH forward, 5'-AAC AGC GAC ACC CAC TCC TC-3'; GAPDH reverse, 5'-GGA GGG GAG ATT CAG TGT GGT-3'. Primers for cytokines were as follows: IL1β-forward, 5'-CAG AAA ACA TGC CCG T-3', IL1β reverse, 5'GCA CTA CCC TAA GGC AG-3', IL6 forward, 5'-CCT GAC CCA ACC ACA AA-3', IL6 reverse, 5'-AGT GTC CTA ACG CTC ATA C-3', IL8 forward, 5'-AGA CAG CAG AGC ACA CAA GC-3, IL8 reverse, 5'-ATG GTT CCT TCC GGT GGT-3', HPRT forward, 5'-GAC TGT AGA TTT TAT CAG ACT GA-3', HPRT reverse, 5'-TGG ATT ATA CTG CCT GAC CAA-3'. PCR was carried out with an initial denaturation step (10 min, 95°C) followed by 40 cycles of denaturation (15 sec, 95°C), annealing (30 sec, 55°C), and extension (30 sec, 72°C). Fluorescence was measured at the end of each extension. Relative mRNA levels were quantified by RQ Study SDS Software v1.3.1 (Applied Biosystems) using the comparative Ct method. The expression level of each gene was normalized to GAPDH levels in each experimental sample. Final data were expressed as mRNA expression in treated cells relative to expression in untreated cells. A melting curve analysis was performed for each amplicon to verify the specificity of each amplification step.

Western blotting

For assaying FZD5 and CaMKII protein expression, cells were lysed with Mammalian Cell Lysis/Extraction reagent (Sigma-Aldrich Chemical Co., Buchs, Switzerland) supplemented with complete mini protease inhibitor cocktail tablets (Roche Diagnostics Schweiz AG, Rotkreuz, Switzerland). After clearing the lysates by high-speed centrifugation, protein concentrations of each sample were determined using a Protein Bradford assay (Bio-Rad Laboratories AG, Reinach, Switzerland). For immunoblotting, 20 µg total protein of each sample was resolved on SDS 4–15% gradient polyacrylamide gels and transferred to PVDF membrane (Millipore AG, Zug, Switzerland). After transfer, the membranes were incubated for 1 h in blocking solution (5% non-fat milk in PBS containing 0.1% Tween-20) and then 1 h with the respective antibody in blocking solution. Antibody binding was detected with a horseradish peroxidase-coupled donkey-anti rabbit secondary antibody diluted 1:10'000 followed by enhanced chemiluminescence (ECL) detection (ECL Plus, Amersham Pharmacia Biotech, Inc., Uppsala, Sweden).

Immunofluorescence staining

For immunofluorescent microscopic analysis of Wnt5A protein expression, macrophages were grown on sterile, endotoxin-free 12 mm glass coverslips pretreated with poly-D-Lysine (Invitrogen AG, Basel, Switzerland). In brief, 100 µL of a 2 x 10^8 cells/mL mononuclear cell suspension were seeded on coverslips in 24-well cluster plates, incubated in IMDM/10% pooled human serum for 2 h under standard cell culture conditions, washed and cultured for 48 h with and without stimuli as described above. After incubation, coverslips were fixed with 3% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100. The coverslips were then exposed to primary antibodies for 60 min, followed by Alexa 594 labeled rabbit anti-goat or Alexa 488 labeled goat anti-rabbit (1:1000: Molecular Probes, Invitrogen Europe), for 60 min. Coverslips were washed three times with PBS, pH 7.4, after each antibody incubation. Nuclei were counterstained with 10 µg/mL diamidino-phenylindole (DAPI; Sigma-Aldrich). Images were taken with an Axioskop 2 microscopy system equipped with an AxioCam MRc digital camera using AxioVision v4.4 software (Carl Zeiss, Feldbach, Switzerland). For quantification of immunofluorescent signals, 5 images were registered for each treatment and were analysed using SigmaScan Pro software (San Jose, CA, USA)..

Immunohistochemical staining

Bone marrow samples were retrieved from the archives of the Department of Pathology of the University Hospital Zurich. They consisted of autopsy samples from patients that had died from severe sepsis or septic shock according to the criteria set forth by the 2003 International Sepsis Definitions Conference[1]. Autopsy samples from patients that had died from gun-shot suicides (age 31–88 yr) served as controls. Microtome sections (4 *µ*m) were cut from formalin-fixed, decalcified and paraffin-embedded marrow samples. Immunohistochemistry was performed on an automated immunohistochemistry module (Bond (TM), Vision BioSystems., Newcastle Upon Tyne, UK) using affinity purified WNT5A polyclonal goat antisera (R&D Systems, Minneapolis; USA), diluted 1: 120 for 60 min followed by the Bond polymer system using diaminobenzidine for visualization. Pretreatment for heat induced epitope retrieval was carried out for 30 min with the EDTA based pH8.8 Bond Epitope Retrieval Solution 2 (Vision BioSystems). Nuclei were counterstained with hematoxylin for bright-field microscopy. Cross-species experiments were performed

to rule out the possibility of non-specific binding of the secondary antibodies. As additional positive and negative controls for Wnt5A immunohistochemistry, we used sections prepared from a 1:10 mixture of regular L-cells (no Wnt5A overexpression) and Wnt5A transfected L-cells (ATCC, CRL-2648/CRL-2814). Cell pellets were formalin-fixed, paraffin-embedded and stained in parallel to the human bone marrow samples.

Quantitation of secreted cytokines

The inflammatory cytokinesIL-1β, IL-6, IL-8, and MIP-1β were quantified in undiluted cell culture supernatants collected 24 h after treatment using the Bio-Plex Human Cytokine Multiplex Assay on the Bio-Plex 2200 platform according to the manufacturer's instructions (Bio-Rad, Hercules, USA). After preparation and culture in IMDM/10% human serum for 24 h, medium was changed to IMDM/2% human serum and cells were treated for an additional 24 h with the stimuli outlined above (see Cell culture section). Complete medium containing 2% human serum was used as a blank. Data were analyzed on the Bio-Plex Reader using Bio-Plex v3.0 software (Bio-Rad, Hercules, USA).

Immunoprecipitation of Wnt5A in sera

Archived serum samples from patients with severe sepsis or septic shock (as above according to the criteria set forth by the 2003 International Sepsis Definitions Conference[1], n = 5; average age 34 years, range 18–62) and from healthy individuals (n = 5; average age 30 years, range 25–45) were stored frozen at –70°C until analysis. None of the patients with sepsis or septic shock had received APC (Drotregocin alpha activated; Xigris™, Eli Lilly, Switzerland).

Aliquots of sera (300 µL) from patients and healthy individuals were incubated with 2 µg of anti-Wnt5A (R&D Systems Europe Ltd., Abington, UK) overnight at 4°C and then adsorbed to protein G beads (Sigma-Aldrich) overnight at 4°C. Samples were washed twice with 1 x IP buffer (Sigma-Aldrich) containing 0.5 M NaCl, four times with 1 x IP buffer and once with 0.1 x IP buffer. Finally, samples were eluted with Laemmli sample buffer (Bio-Rad). The eluate was analyzed by 10% SDS–polyacrylamide gel electrophoresis and Western blot onto PVDF membranes (Millipore) using the same anti-wnt5A antibody and chemiluminometric detection as described above.

To investigate the inflammatory status of the sera and to exclude impaired clearance of Wnt5A , due to renal insufficiency in patients with sepsis we analyzed neopterin, a common inflammatory metabolite released by activated macrophages[2] and serum creatinine. Neopterin was measured by HPLC for pteridins after oxidation as described previously[3].check references Creatinine was measured by ISO certified analysis using kinetic Jaffe reaction and a Beckman Unicel DXC 600 analyser. Protein was measured by Protein Bradford assay (BioRad).

Generation of small interfering RNA (siRNA) and transfection of macrophages.
Template DNA probe specific for human Wnt5A (RZPDp3000B068D) was obtained from RZPD (Berlin, Germany). From this probe, double-stranded RNA was synthesized with T7 enzyme, and 22 bp small interfering RNA probes (siRNA) directed against Wnt5A were prepared by dicing using the X-tremeGENE siRNA Dicer kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. The siRNA was column-purified and RNA concentrations were measured with a NanoDrop spectrophotometer. Human macrophages were transfected for 5 hr at 37°C with 2.0 µg siRNA with the X-tremeGENE siRNA transfection reagent (Roche Diagnostics) in IMDM with 10% human pooled serum. Cells were then washed and incubated in IMDM/10% human pooled serum with 100 U/mL INFγ and 1 ng/mL LPS for 8 h (optimal time for mRNA expression) or 24 h (optimal time point for protein secretion). siRNA directed against luciferase GL3 duplex (Dharmacon, Lafayette, USA) served as both transfection and negative control. To ensure specificity of the observed effect of siRNA directed against Wnt5A, we measured expression of the housekeeping gene HPRT using the primers and PCR conditions as described above.

References

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3. Franscini N, Blau N, Walter RB, Schaffner A, Schoedon G. Critical role of interleukin-1beta for transcriptional regulation of endothelial 6-pyruvoyltetrahydropterin synthase. Arterioscler Thromb Vasc Biol. 2003;23:e50-53.

RESULTS

FIGURE I

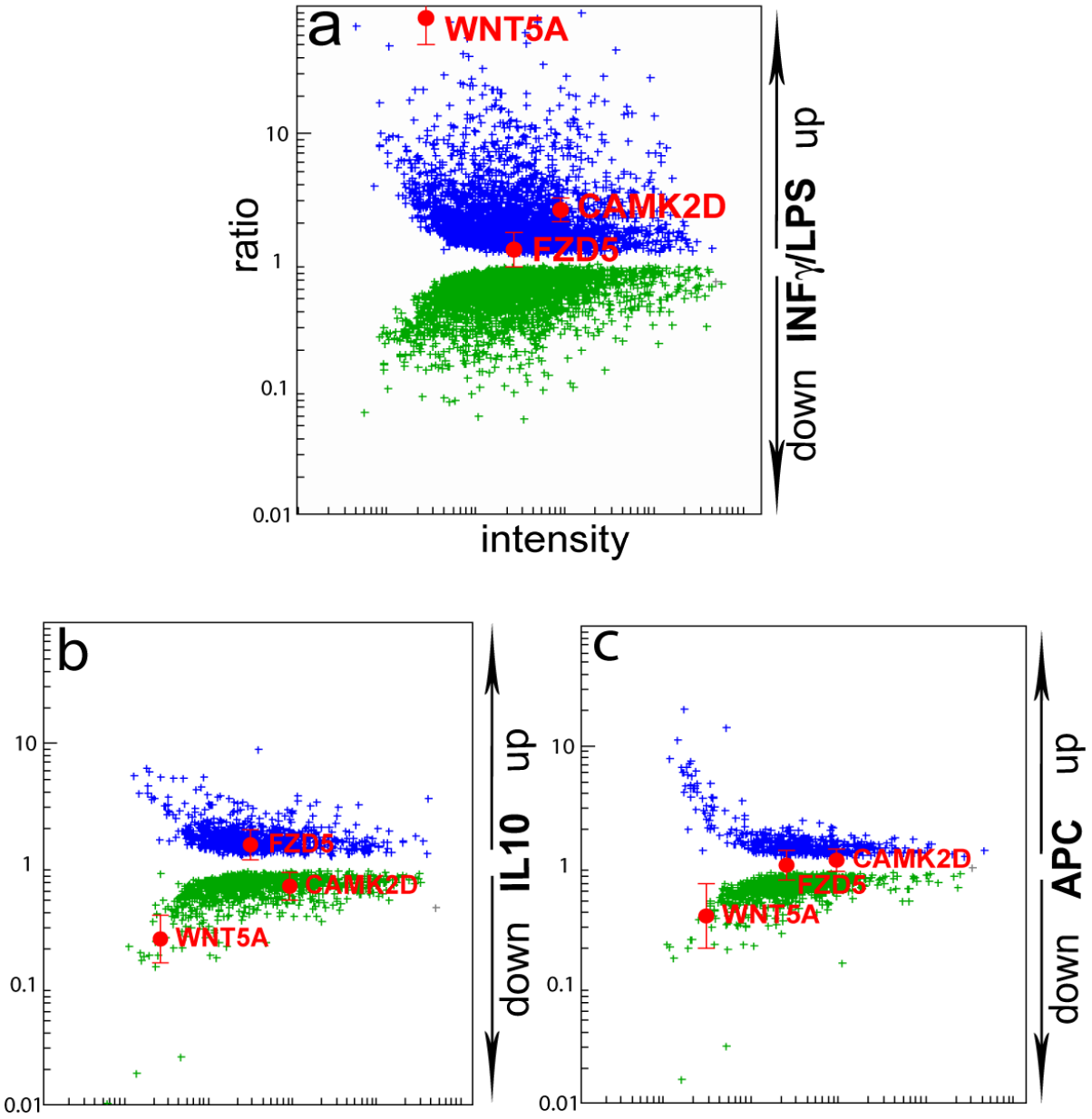

Figure I. Gene expression profile analysis of human macrophages.

Human macrophages were cultured as described in Methods. They were treated with LPS (10 ng/mL) and INFγ (100 U/mL) for the last 8 h in culture and the differential mRNA transcription pattern was characterized by oligonucleotide microarray with Agilent 44k gene chips and analysis using Rosetta Biosoftware as described in supplemental material. The ratio plot displays combined results from three

independent experiments (mean ± SD of selected genes). The x-axis (Log intensity) shows baseline gene expression and the y-axis (Log ratio) shows the ratio of the signal intensities in the two hybridized samples. Individual genes are represented as blue (significantly upregulated, $p \leq 0.001$), green (significantly downregulated, $p \leq 0.001$). Selected genes (Wnt5A, CaMKII and FZD5) are shown in red. (**b**) Re-ratio plot of human macrophages treated with LPS/INFγ and IL-10 (5 ng/mL) versus human macrophages treated with LPS/INFγ. Plots represent combined results from three independent experiments, each. The re-ratio analysis tool of Rosetta Biosoftware was used for direct comparison between two samples that were hybridized against a common reference. (**c**) Re-ratio plot of human macrophages treated with LPS/INFγ and APC (5 µg/mL) versus human macrophages treated with LPS/INFγ for 8h.

Table I

Selected genes related to inflammation that are upregulated in human macrophages treated with LPS/INFγ. Influence of IL10 or APC on the expression of these genes.

Sequence Name(s)	Accession #	Sequence Description	Fold Change		
			LPS/INFγ vs Untreated control	LPS/INFγ/IL10 vs LPS/INFγ #	LPS/INFγ/APC vs LPS/INFγ #
IL1B	NM_000576	Homo sapiens interleukin 1, beta	+87.9	-1.5	+1.2
WNT5A	NM_003392	Homo sapiens wingless-type MMTV integration site family, member 5A	+79.2	-4.2	-2.7
CCL2	NM_002982	Homo sapiens chemokine (C-C motif) ligand 2	+17.1	-1.1	-1.0
IL6	NM_000600	Homo sapiens interleukin 6 (interferon, beta 2)	+15.3	-6.0	-1.8
CCL4	NM_002984	Homo sapiens chemokine (C-C motif) ligand 4	+13.9	-1.6	+1.0
IL8	NM_000584	Homo sapiens interleukin 8	+3.6	-1.9	-1.9
CaMK2D	NM_172127	Homo sapiens calcium/calmodulin-dependent protein kinase (CaM Kinase) II delta	+2.4	-1.5	+1.0
FZD5	NM_003468	Homo sapiens frizzled homolog 5 (Drosophila)	+1.2	+1.4	-1.0

Fold changes (up or down) of gene expression were calculated using the ratio or re-ratio# function of Rosetta Biosoftware that allows direct comparison between two samples that were both hybridized against a common reference. Data are from three independent experiments, $p < 0.001$.

Table II

Wnt family members and Wnt pathway related gene expression in human macrophages treated with LPS/ INFγ versus untreated cells (p<0.001)

Sequence Name(s)	Accession #	Sequence Description	Fold Change
WISP3	NM_130396	Homo sapiens WNT1 inducible signaling pathway protein 3	+1.6
WNT8A	NM_031933	Homo sapiens wingless-type MMTV integration site family, member 8A	-1.2
WNT7B	NM_058238	Homo sapiens wingless-type MMTV integration site family, member 7B	-1.1
WNT2	NM_003391	Homo sapiens wingless-type MMTV integration site family member 2	-1.1
WNT8B	NM_003393	Homo sapiens wingless-type MMTV integration site family, member 8B	+1.0
WIF1	NM_007191	Homo sapiens WNT inhibitory factor 1	+1.2
WNT4	NM_030761	Homo sapiens wingless-type MMTV integration site family, member 4	+1.2
WNT1	NM_005430	Homo sapiens wingless-type MMTV integration site family, member 1	-1.0
WNT9A	NM_003395	Homo sapiens wingless-type MMTV integration site family, member 9A	-1.9
WNT10A	NM_025216	Homo sapiens wingless-type MMTV integration site family, member 10A	-1.2
WNT7A	NM_004625	Homo sapiens wingless-type MMTV integration site family, member 7A	-1.8
WISP2	NM_003881	Homo sapiens WNT1 inducible signaling pathway protein 2	-1.1
WNT10A	NM_025216	Homo sapiens wingless-type MMTV integration site family, member 10A	-1.2
WNT5B	NM_030775	Homo sapiens wingless-type MMTV integration site family, member 5B (WNT5B)	-1.2
WNT11	NM_004626	Homo sapiens wingless-type MMTV integration site family, member 11	+1.1
WISP1	NM_003882	Homo sapiens WNT1 inducible signaling pathway protein 1	-1.1
WNT6	NM_006522	Homo sapiens wingless-type MMTV integration site family, member 6	-1.0
WNT3A	NM_033131	Homo sapiens wingless-type MMTV integration site family, member 3A	+1.1
WNT3	NM_030753	Homo sapiens wingless-type MMTV integration site family, member 3	-1.2
WNT9B	NM_003396	Homo sapiens wingless-type MMTV integration site family, member 9B	+1.0
WNT10B	NM_003394	Homo sapiens wingless-type MMTV integration site family, member 10B	-1.1
WNT2B	NM_004185	Homo sapiens wingless-type MMTV integration site family, member 2B	-1.9
WNT16	NM_057168	Homo sapiens wingless-type MMTV integration site family, member 16	+1.0
WNT7B	NM_058238	Homo sapiens wingless-type MMTV integration site family, member 7B	+1.3
WNT5A	NM_003392	Homo sapiens wingless-type MMTV integration site family, member 5A	+79.2

DETAILED FIGURE LEGENDS

Figure 1. Wnt5A expression is induced by LPS/INFγ and TLR agonists and is blocked by IL-10 and APC.

(**a**) mRNA expression of Wnt5A measured by quantitative RT-PCR. macrophages were unstimulated (control) or treated with LPS, INFγ, IL-10, and APC, as indicated. Changes in mRNA expression were normalized to changes in GAPDH expression and are expressed as mean ± SD from three independent experiments. (**b**) mRNA expression of Wnt5A measured by quantitative RT-PCR. Macrophages were unstimulated (control) or treated with TLR agonists as follows: 5 μM oligodeoxynucleotides (ODN, TLR9), 0.5 μg/mL ssRNA40/LyoVec (TLR8), 0.5 μg/mL Imiquimod (TLR7), 500 ng/mL FSL1 (TLR6/2), 5 μg/mL Flagellin (TLR5), 25 μg/mL Poly I:C (TLR3), 10^8 cells/mL HKLM (TLR2), and 0.5 μg/mL PamCSK4 (TLR1/2), 10 ng/mL *E.coli* K12 LPS (TLR4), (tlrl-kit, InvivoGen, USA). Data is expressed as described in (a). (**c**) Immunofluorescence staining of Wnt5A protein in macrophages. Cells grown on coverslips were treated for 24 h with LPS/INFγ, LPS/INFγ/IL-10 or LPS/INFγ/APC, in the same concentrations as used for microarray experiments. Fluorescence was visualized using a Zeiss Axioskop 2 fluorescence microscope and an AxioCam MRc digital camera using Axiovision v4.4 software. All photomicrographs within a series were taken using the same exposure time and frame. Magnification, 630X. (**d**) Quantification of the fluorescence signal intensity of Wnt5A. Bars represent total intensity per cell count (mean±S.E.M, n=15 pictures). $p<0.05$ (one-way ANOVA).

Figure 2. The Wnt5A signaling pathway is active in macrophages and is blocked by sFRP1.

(**a**) Detection of CaMKII and FZD5 protein in lysates of cultured macrophages treated with LPS, INFγ, IL-10 and APC, as indicated, for 24 h. (**b**) Immunofluorescent detection of phosphorylated CaMKII. Macrophages were cultured for 24 h prior to incubation for 30 min or 120 min with LPS, INFγ, sFRP1 or Wnt5A, as indicated. Control cells were untreated. Wnt5A and sFRP1 were mixed and pre-incubated for 30 min before addition to the cells. Phosphorylated CaMKII was detected using a specific anti-active CaMKII antibody (see Methods). Fluorescence was visualized using a Zeiss Axioskop 2 fluorescence microscope and an AxioCam MRc digital

camera using Axiovision v4.4 software. All photomicrographs within a series were taken using the same exposure time and frame. Magnification, 630X. (**c**) Quantification of the fluorescence signal intensity of phosphorylated CaMKII. Bars represent total intensity per cell count (mean±S.E.M, n=10 pictures). $p<0.05$ (one-way ANOVA).

Figure 3. Induction of inflammatory cytokine secretion by Wnt5A signaling, its modulation by anti-inflammatory mediators IL-10 and APC.

Concentrations of IL-6, IL-8, IL-1β and MIP-1β protein secreted by macrophages treated with Wnt5A in the absence and the presence of IL-10 or APC, respectively. Control cells were untreated. Cytokines were measured in cell culture supernatants collected 24 h after treatment using the Bio-Plex Human Cytokine Multiplex Assay on the Bio-Plex 2200 platform. Cytokine concentrations in treated cells were normalized to the concentrations in control cells and are presented as mean ± SEM from three independent experiments. *$p<0.05$.

Figure 4. Effect of blocking Wnt5A signaling with siWnt5A on inflammatory cytokines secretion

(**a**) Left graph, fold induction of Wnt5A mRNA expression in LPS/INFγ stimulated macrophages after transfection with siWnt5A. Right graph, HPRT mRNA expression in LPS/INFγ stimulated macrophages after transfection with siWnt5A The expression level was normalized to GAPDH mRNA in each experimental sample. Data are presented as mRNA expression in stimulated siWnt5A-(open bar) and mock-transfected (black bar) cells relative to the expression level in unstimulated transfected cells. Results are expressed as mean ± SEM of three independent experiments. *$p<0.05$ (**b**) IL-6, IL-8, IL-1β mRNA expression and secretion by LPS/INFγ stimulated macrophages after transfection with siWnt5A. Data are presented as mRNA expression in stimulated siWnt5A-(open bar) and mock-transfected (black bar) cells relative to the expression level in unstimulated transfected cells, and as the ratio of the concentration of cytokine secreted by stimulated cells to that secreted by unstimulated cells (in siWnt5A or mock transfected cells). Results are expressed as mean ± SEM of three independent experiments. *$p<0.05$ (**c**) Concentrations of IL-6, IL-8, IL-1β and MIP-1β secreted by macrophages. Cells were stimulated with LPS/INFγ in the absence or presence of

sFRP1. Control cells were untreated. Cytokine concentrations in treated cells were normalized to the concentrations in control cells and are presented as mean ± SEM from three independent experiments. *p<0.05.

Figure 5. Wnt5A protein is present in the serum and bone marrow of patients with severe sepsis or septic shock.

(**a**) Immunoprecipitation of Wnt5A in serum from patients with sepsis (lanes 5–8) and from healthy individuals (lanes 1–4). Lane 9 shows Wnt5A conditioned medium (Wnt5A-CM) that was immunoprecipitated following the same protocol. The molecular weight of the band visualized corresponds to ~40 kDa, as expected for Wnt5A. (**b**) Left graph, densitometric analysis of bands detected by immunoprecipitation using a ChemiDoc XRS system and Quantity One v4.5.0 software (Bio-Rad). The average density is normalized to the total protein concentration (mg/mL) of each individual sample. Right graph, serum neopterin concentrations (nmol/L) normalized to the respective creatinine concentration (µmol/L) in the samples used for Wnt5A immunoprecipitation. Data are from one representative of four independent experiments. p<0.05 (**c**) Wnt5A in sections from bone marrow biopsies of patients with fatal sepsis and from healthy individuals[17] was detected by immunohistochemistry as described in Methods. As controls, sections of L-cells and Wnt5A cells mixed in a ratio of 10:1 were treated in the same manner. Photomicrographs were acquired with a Zeiss Axioskop 2 equipped with Axiocam MRc digital camera and Axiovision v4.4 software. Magnification, 400X (left panel) and 640X (right panel). Arrows point to Wnt5A positive macrophages.

7. Conclusions

APC has previously been shown to have anti-inflammatory effects on several cell types. However the exact mechanism of its anti-inflammatory action is still unknown. The results presented herein expand our knowledge on targets of APC and opens new lines of investigation to be explored in order to elucidate the unique properties of this molecule.

In a genome wide transcriptional screen of macrophages stimulated with LPS and IFN-γ, we have identified Wnt5A as one of the most highly induced genes. Suppression of inflammation-forced Wnt5A expression by APC implicated Wnt5A in an active role in the inflammatory response. We confirmed regulation of Wnt5A protein in macrophages and detected it in sera and bone marrow macrophages of patients with severe sepsis. We established that a functional Wnt5A/frizzled-5/CaMKII signaling pathway was essential for macrophage inflammatory activation. To prove the essential contribution of Wnt5A we measured inflammatory cytokines after stimulation with Wnt5A, silenced Wnt5A by siRNA, and blocked receptor binding with soluble Frizzled–related peptide-1 (sFRP1). This results showed that Wnt5A is critically involved in inflammatory macrophage signaling in sepsis and is a target for anti-inflammatory mediators like APC or antagonists like sFRP1.

In conclusion this work adds new important elements to the comprehension of macrophage activation during local and systemic inflammation, shows new targets for the anti-inflammatory action of APC and may open new therapeutical ways to the management of inflammation.

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To my parents, for believing…